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PRINCIPAL INVESTIGATOR: Oscar U. Scremin, M.D., Ph.D.
Tsung-Ming Shih, Ph.D.
Donald J. Jenden, M.D., Ph.D.

CONTRACTING ORGANIZATION: Brentwood Biomedical Research Institute
Los Angeles, California 90073

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6. AUTHOR(S) :
Oscar U. Scremin, M.D., Ph.D.
Tsung-Ming Shih, Ph.D.
Donald J. Jenden, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brentwood Biomedical Research Institute
Los Angeles, California 90073

Email: oscremin@ucla.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This report describes the effects of treatment with low levels of the cholinesterase (ChE) inhibitors Sarin (0.5 LD50 s.c. 3 times weekly) and pyridostigmine bromide (PB, 80 mg/L in drinking water) alone or in combination for 3 weeks as compared with untreated controls. At 2, 4 and 16 weeks after exposure, we studied the brain regional levels of glucose utilization (rCGU), as well as electroencephalographic activity, heart rate and locomotor activity with a telemetry system, on an hourly basis throughout the day, during seven-day periods. Statistical parametric maps of cerebral cortex rCGU obtained were compared with similar maps of cortical cerebral blood flow (rCBF) obtained during the previous year. The slope relating rCBF to rCGU in cerebral cortex was enhanced by sarin and depressed by sarin + PB four weeks after treatment. The analysis of cardiovascular regulation indicated significant reduction of heart rate and enhanced heart rate variability during PB treatment that recovered partially one week after treatment. Sarin (4 weeks) and PB (4 and 16 weeks) also showed enhanced HR variability. Sarin induced enhanced heart rate variability at all times after treatment. In conclusion, the changes in rCBF/rCGU coupling observed 4 weeks after sarin are consistent with residual cholinesterase inhibition in vascular compartments. The changes in heart rate variability deserve further exploration.

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INTRODUCTION.

Organophosphorus (OP) cholinesterase (ChE) inhibitors are among chemical weapons to which army personnel and civilians could be exposed, at symptomatic or sub-symptomatic doses. The carbamate ChE inhibitor pyridostigmine bromide (PB) has been fielded as a prophylactic treatment against OP ChE inhibitors by the US Armed Forces and used in the Persian Gulf War (Dirnhuber et al., 1979); (Leadbeater et al., 1985); (Koplovitz et al., 1992); (Kluwe et al., 1987); (Keeler et al., 1991). Although acute intoxication with OP ChE inhibitors and the protective effect of PB on this phenomenon have been extensively studied in animals (Ecobichon and Joy, 1982); (Sidell, 1974); (Chambers, 1992), the potential long term harmful effects of low level (subsymptomatic) exposure to OP ChE inhibitors, alone or in combination with PB have received little attention. This is the objective that the present proposal intends to address.

In our experimental approach to this objective, we are evaluating the possible occurrence of delayed neurologic dysfunction after exposure of animals to PB or to doses of the OP cholinesterase inhibitor sarin, low enough to be free of acute toxic effects, alone or in combination with PB treatment. During the first year of support, inhibited (passive) avoidance and open field activity were used to assess cognitive function, motor activity, and habituation. Auditory startle and nociceptive threshold were assessed to determine the existence of possible neurological dysfunction. In addition, we analyzed, in key brain regions, the activity of ChAT and AChE, the enzymes responsible for ACh synthesis and degradation respectively, as well as the expression of muscarinic

cholinergic receptors. These assays were performed in the same animals that were subjected to the neurobehavioral tests mentioned above.

These studies were preceded by experiments aimed at establishing the optimal doses of sarin and PB. For sarin, the optimal dose was defined as the highest dose not associated with toxic signs following single or multiple doses within the three week period of treatment. In the case of PB, the optimal dose was defined as one producing 20-30% inhibition of plasma butyrylcholinesterase (BuChE). This is the degree of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf war (Keeler, Hurst, and Dunn, 1991) (90 mg PB over 24 hrs, divided in three oral doses).

During the second year of support, we continued the study of cognitive function after exposure to subtoxic doses of cholinesterase inhibitors with the same experimental design described above, using the conditioned avoidance test. In addition, the possible existence of neurologic dysfunction in the exposed animals was tested by a study of the baroreceptor reflex, a well characterized autonomic nervous system regulatory mechanism that includes peripheral as well as central cholinergic mechanisms (Higgins et al., 1973) (Brezenoff and Giuliano, 1982). The effects of pharmacological challenges that increased or decreased arterial blood pressure acutely was quantified to characterize the gain of the baroreceptor reflex and the incidence of heart arrhythmias. Finally, regional cerebral blood flow (rCBF) was measured with the Iodo-^{14}C- antipyrine technique in order to produce cerebral functional activation maps.

During the third year of support, the object of the current report, we studied the brain regional levels of glucose utilization, as well as electroencephalographic activity, heart rate and locomotor activity with a telemetry system in animals exposed to PB, sarin, sarin plus PB, and untreated controls. The telemetry system measurements were performed on an hourly basis throughout the day, during seven-day periods. Statistical parametric maps of cerebral cortex CGU obtained during this period of support were compared with similar maps of cortical cerebral blood flow obtained during the second year of support that had shown significant alterations four weeks after treatment with sarin. The analysis of cardiovascular regulation reported previously (2002 Annual Report) was continued with the analysis of heart rate power spectra, an important physiological parameter sensitive to alterations in cholinergic tone (Cerutti et al., 1991).

MATERIALS AND METHODS.

1. Animals.

Male Crl:CDBR Vaf/Plus Sprague-Dawley rats, weighing 250-300g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12- h light-dark full spectrum lighting cycle with lights on at 0700 h. Laboratory chow and water were freely available. Research was conducted in compliance with the Animal Welfare Act and

other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2. Materials.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical and Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml/kg subcutaneously. PB was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared twice weekly in tap water at a concentration of 80 mg/L and provided as drinking water to experimental groups for a three-week period.

3. Experimental Procedures.

Animals were exposed to treatments (saline, sarin, PB or sarin+PB) during three weeks at the US Army Institute of Chemical Defense laboratory in Aberdeen Proving Ground (APG). After a period of 1 to 15 weeks following treatment, depending on the experimental groups, they were transported by air-conditioned vans and air-freight to the Veterans Affairs Greater Los Angeles Healthcare System (VA GLAHS) laboratory were

they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables.

Anesthesia for surgical procedures: Animals were anesthetized by exposure to 2.5% halothane in air in a closed plexi-glass chamber with continuous flow of gas from an anesthesia machine. After 2-3 minutes the animal was transferred to a table provided with a heating pad, and a maintenance concentration of halothane (1.5%) was given by mask throughout the surgical procedure. A scavenging system (Fluosorb) prevented excess halothane from reaching the environment. The concentration was raised if withdrawal to painful stimulation was observed. Anesthesia was discontinued after surgical wounds were sutured. The condition of the animal was monitored frequently during the post-operatory period.

Implantation of telemetry transducers: Radiotelemetry transmitters, specifically designed for rats (Data Sciences International TL10M3 F50 EEE) were implanted subcutaneously on the back of the animal, approximately just below the shoulder blades, using aseptic technique. The transmitter weighs 11.5 g., it has a volume of 5.5 cm^3 and three pairs of leads. Surgical instruments were sterilized using dry heat (glass bead sterilizer, Germinator 500, Cell Point Scientific Inc, Rockville, MD). Stainless steel screws, radiotelemetry implant and leads were sterilized by immersion overnight in Cydex (glutaraldehyde) followed by rinsing with sterile saline. Instruments, radiotelemetry implant, leads, screws and pads of sterile gauze, and suture material were placed on a towel previously autoclaved. The surgeon wore sterile surgeons gloves, mask

and a clean laboratory coat. The skin over and around the implantation site was shaved closely (against the grain) with a # 40 blade, and scrubbed with sudsing povidone iodine (Betadine) in a spiral pattern starting at the incision site and moving outwards. The site was then rinsed with alcohol, followed by a final application of a non-sudsing povidone-iodine solution. A 2 cm skin incision was performed between the scapulae. The radiotelemetry implant was placed on a pocket fashioned by blunt dissection of the subcutaneous space at the site. A second 1.5 cm incision was performed at the midline of the cranium between 5 mm rostral and 10 mm caudal to bregma. The skin was treated as described above for the scapular site prior to incision. Two pairs of leads from the radiotelemetry implant were tunneled under the skin and connected by twisting to four stainless steel 1/16" screws (Small Parts MX-0090-1B) inserted in the cranium at a depth just enough to touch (without penetrating) the duramater. Screws were inserted at the following coordinates: Bregma (distance to bregma with positive values representing distance on the rostral, and negative distance on the caudal, direction) 1mm, lateral 2mm; Bregma –2mm, lateral 5mm; Bregma –4mm, lateral 7mm; and Bregma –7mm, lateral 3mm. One additional pair of leads was placed by suturing with 5-0 polyvinyl material to the subcutaneous tissue over the right scapula and the heart apex for recording of electrocardiogram (ECG). The skin incision over the radiotelemetry implant was closed with 5-0 polyvinyl suture material.

Measurement of cerebral glucose utilization: Regional cerebral glucose utilization (rCGU) was measured with the ^{14}C 2-deoxyglucose (DG) autoradiographic technique (Sokoloff et al., 1977). One arterial and one venous catheter were implanted in the

femoral vessels under halothane anesthesia used as described above. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for one hour. In these cages the animals rest in prone position with their limbs hanging to the sides. Acrylic non-traumatic bars entrap the animal preventing locomotion but allowing limb and head movements. The cage was covered with a cloth in order to prevent cooling of the animal and to eliminate visual contact with the environment. Rectal temperature was recorded with a BAT-12 thermocouple thermometer connected to a TCAT-1A (Physitemp, Inc.) temperature controller and a source of radiant heat. A sample of arterial blood was obtained for measurement of blood gases and pH in a Radiometer ABL-5 blood acid-base system and then ^{14}C 2-DG (Amersham Corp., Arlington Heights, IL) dissolved in 0.5 ml of saline at a concentration of 100 μCi/kg body mass was administered intravenously at a rate of 1 ml/min for 30 seconds. Eleven arterial blood samples (70 μl) were then obtained over a period of 45 min for measurement of glucose concentration (glucose oxidase method) and radioactivity (liquid scintillation counting) to allow calculation of rCGU. After euthanasia (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus), performed immediately after obtaining the last blood sample (45 min after ^{14}C 2-DG infusion) the brain was removed, flash frozen in methylbutane chilled to -70°C and embedded in OCT compound for later sectioning in a cryostat at -20°C in 20 μm slices. These sections were heat dried and exposed to Kodak Ektascan film in spring-loaded X-ray cassettes along with 8 standards of known radioactivity to obtain an ^{14}C-2-deoxyglucose autoradiograph. Tissue radioactivity was derived by densitometry of tissue and standards autoradiographs and rCGU values were obtained using the operational

equation and values for the lumped and rate constants previously described (Sokoloff, Reivich, Kennedy, Des Rosiers, Patlak, Pettigrew, Sakurada, and Shinohara, 1977).

4. Experimental Design.

4.1. Experimental groups.

Separate sets of animals were studied 2, 4, and 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Number of animals was 12 per treatment group, as determined by statistical power analysis, and the total number of groups (treatments x times after treatments) 12, with a grand total of 144 rats.

Treatment group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline. Treatment group 2 animals received PB in drinking water (80 mg/L) and were injected with saline. Treatment group 3 animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to 0.5 LD50). Treatment group 4 animals received PB in drinking water and were injected with sarin. PB in drinking water was provided continuously to groups 2 and 4 animals starting on Monday morning at 0800 hour. At 0900 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week for three weeks in groups of 4 animals per dose. PB in drinking was terminated and switched to regular tap water at 1700 hour on Friday of the third week. Animal dosing procedures were performed at the APG laboratory. All animals were then shipped to the VA GLA

laboratory location, where the planned main biochemical and behavioral studies in these animals were performed 2 weeks, 1 month, and 4 months after sarin, sarin + PB, PB, or control treatments.

One additional group of five animals was treated with PB in the drinking water after they had been implanted with radiotelemetry transducers. Four days after implantation, recording of bioelectric parameters started and continued for one week prior to PB administration, a second week during PB administration, and a third week after discontinuation of PB.

4.2. Data Analysis.

Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data is presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) at each interval after exposure to drugs or saline with one factor (treatment) at four levels (saline, PB, sarin, sarin+PB). This analysis was followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method or Bonferroni tests adjusted for three comparisons.

In order to analyze circadian variations of heart rate and locomotor activity (LA), as well as heart rate variability, ECG and LA were recorded every hour, for an interval of 300 seconds, during seven consecutive days, starting four days after implantation of

telemetry units. Using the Data Sciences software, the time of occurrence of each heartbeat was extracted from the raw ECG and a 300 seconds time series of consecutive inter-beat intervals (RR intervals) was constructed to allow subsequent time domain and frequency domain measurements.

Time Domain: R-R intervals statistic parameters were calculated over windows of 2 seconds duration. For that, each 300 seconds data set was segmented in non-overlapping windows of 2 sec. and for each window, the mean and the standard deviation of the RR intervals ("RR mean" and "RRSD") were computed. For each hourly segment the average value of these two parameters were calculated. In addition the average for the entire of diurnal and nocturnal period (n=12 in both cases) were computed.

Frequency domain: For this analysis, the time series of RR intervals was re-sampled at a rate of 6 Hz and subsequently the frequency spectrum was calculated using a Fast Fourier Transform that uses routines written in Matlab (The Mathworks, Inc.). The spectrum was further averaged over consecutives non-overlapping windows of 128 data points (leading to a window length of 21.33 seconds and a lowest frequency resolved of 0.05 Hz).

RESULTS.

Cerebral glucose utilization.

The values of body mass, arterial blood gases, body temperature, and plasma glucose concentration in all experimental groups are presented in Table 1. No significant differences were detected in any of these variables among experimental groups within a given time after treatment.

Table 1. Physiological variables during glucose utilization measurements.

TREATMENT		Control	PB	Sarin	Sar+PB	Control	PB	Sarin	Sar+PB	Control	PB	Sarin	Sar+PB
TIME AFTER TR.		2 WEEKS				4 WEEKS				16 WEEKS			
pH	MEAN	7.464	7.435	7.456	7.458	7.456	7.443	7.451	7.463	7.442	7.440	7.453	7.464
-Log [H^+]	SE	0.008	0.006	0.010	0.002	0.009	0.009	0.005	0.004	0.012	0.007	0.005	0.017
pCO2	MEAN	41.80	43.13	40.40	42.00	41.29	42.28	39.88	40.83	42.00	42.00	42.83	41.80
mm Hg	SE	1.24	1.81	1.03	0.71	1.48	0.93	0.97	0.95	1.06	0.93	0.65	2.35
pO2	MEAN	84.20	81.50	83.60	85.00	81.43	86.94	83.31	86.17	85.00	83.92	83.00	83.40
mm Hg	SE	0.86	0.29	1.75	2.04	1.89	1.21	0.71	2.18	2.84	2.82	2.21	1.17
Body mass	MEAN	489.20	489.50	464.60	459.00	515.86	522.33	506.00	509.00	633.83	703.50	645.67	636.00
(g)	SE	16.95	15.04	18.99	24.24	13.16	17.14	19.08	19.13	22.13	42.32	27.56	27.45
Temp(i)	MEAN	37.82	37.88	38.12	38.10	37.87	37.42	37.83	37.80	37.52	37.63	37.68	37.44
°C	SE	0.25	0.35	0.07	0.25	0.22	0.33	0.17	0.25	0.21	0.16	0.15	0.32
Temp(f)	MEAN	37.86	37.95	37.92	38.10	37.96	37.60	37.83	37.77	37.77	37.78	37.85	37.84
°C	SE	0.19	0.19	0.11	0.28	0.11	0.25	0.15	0.23	0.15	0.22	0.20	0.31
P. Glucose(i)	MEAN	10.08	9.39	10.25	8.84	9.91	10.61	9.50	10.22	10.60	9.93	9.62	10.59
mM	SE	0.62	0.42	0.69	0.13	0.64	0.61	0.40	0.68	0.30	0.62	0.68	0.46
P. Glucose(f)	MEAN	9.92	9.98	10.15	9.40	10.08	10.82	9.92	10.06	10.07	10.08	9.71	10.50
mM	SE	0.32	1.05	0.65	0.33	0.37	0.76	0.41	0.43	0.27	0.64	0.62	0.52

Similar values of temperature and plasma glucose concentration at the beginning (P. Glucose(i), Temp(i)), and at the end (P. Glucose(f), Temp(f)) of the experiment, respectively, indicated the existence of steady state conditions.

The time course of plasma glucose concentration and ^{14}C-2DG related radioactivity throughout the experiments are shown in Fig. 1. Plasma glucose values were stable and the kinetics of plasma ^{14}C-2DG followed the expected time course with the highest values attained during the short infusion period, followed by a decay to very low levels at the end of the experimental period.

Measurements of rCGU were performed with the ^{14}C-2DG quantitative autoradiographic technique (see methods). Three hundred cerebral cortical regions were sampled in 15 coronal planes, identified according to the Atlas of Paxinos and Watson in Fig 2 within amygdala (Am), auditory cortex (Au), primary auditory cortex (Au1), barrel cortical field (BF), ectorhinal cortex (Ect), entorhinal cortex (Ent), face cortical area (Fa), forelimb cortical area (FL), hindlimb cortical area (HL), insular cortex (I), primary motor cortex (M1), secondary motor cortex (M2), parietal association area (PA), piriform cortex (Pir), retrosplenial cortex (RS), primary somatosensory cortex (S1), secondary somatosensory cortex (S2), temporal cortex (Te), trunk cortical area (Tr), primary visual cortex (V1), and secondary visual cortex (V2). Means of CBF for every region are displayed in Fig 2 in color coded maps in which the ordinate represents distance along the rostro-caudal axis of the brain, the abscissa position of regions relative to the midline, and mean rCGU of every cell is represented on a color scale. Statistical significance against the control group was tested by ANOVA and Bonferroni tests adjusted for three contrasts.

The analysis of cerebral cortical distribution of rCGU has been completed for the 4 week post-treatment period. This is an important milestone because results on this program

reported the previous year had indicated an increase in cortical blood flow (rCBF) in sarin treated animals at this time. The presently reported results indicate that rCGU in sarin treated animals at 4 weeks post-treatment is not different from untreated controls in any of the cortical regions (Fig 2). The same lack of significant changes with regards to controls holds true for the other two treatments for which the analysis has been performed (PB and Sarin+PB) as shown in Fig. 2.

Dependence of cerebral blood flow on cerebral metabolism was tested by regression analysis of rCBF on rCGU using paired mean values of those variables for each of the cortical regions studied. A strong dependence was found, but the regression slope of sarin treated animals was significantly higher, and that of sarin + PB treated animals significantly lower, than controls. The regression slope of PB treated animals was not significantly different from controls (Tables 2-5, Figs 3-4).

Table 2. Regression Estimation for rCBF on rCGU (4 week Control).

	Intercept	Slope
Parameter	**B(0)**	**B(1)**
Regression Coefficients	0.2227	0.8634
Lower 95% Confidence Limit	0.0533	0.7475
Upper 95% Confidence Limit	0.3922	0.9793
Standard Error	0.0865	0.0591
Standardized Coefficient	0.0000	0.6465
T Value	2.5761	14.6031
Prob Level (T Test)	0.0105	0.0000
Reject H0 (Alpha = 0.0500)	Yes	Yes
Power (Alpha = 0.0500)	0.7283	1.0000

Table 3. Regression Estimation for rCBF on rCGU (4 week Sarin).

Parameter	Intercept B(0)	Slope B(1)
Regression Coefficients	0.5829	1.3116
Lower 95% Confidence Limit	0.2534	1.0402
Upper 95% Confidence Limit	0.9124	1.5831
Standard Error	0.1681	0.1385
Standardized Coefficient	0.0000	0.4829
T Value	3.4670	9.4706
Prob Level (T Test)	0.0006	0.0000
Reject H0 (Alpha = 0.0500)	Yes	Yes
Power (Alpha = 0.0500)	0.9326	1.0000

Table 4. Regression Estimation for rCBF on rCGU (4 week PB).

Parameter	Intercept B(0)	Slope B(1)
Regression Coefficients	0.1999	0.7726
Lower 95% Confidence Limit	0.0087	0.6603
Upper 95% Confidence Limit	0.3911	0.8850
Standard Error	0.0975	0.0573
Standardized Coefficient	0.0000	0.6154
T Value	2.0495	13.4784
Prob Level (T Test)	0.0413	0.0000
Reject H0 (Alpha = 0.0500)	Yes	Yes
Power (Alpha = 0.0500)	0.5331	1.0000

Table 5. Regression Estimation for rCBF on rCGU (4 week Sarin + PB).

Parameter	Intercept B(0)	Slope B(1)
Regression Coefficients	0.9250	0.3845
Lower 95% Confidence Limit	0.7541	0.2690
Upper 95% Confidence Limit	1.0959	0.5001
Standard Error	0.0872	0.0590
Standardized Coefficient	0.0000	0.3534
T Value	10.6081	6.5222
Prob Level (T Test)	0.0000	0.0000
Reject H0 (Alpha = 0.0500)	Yes	Yes
Power (Alpha = 0.0500)	1.0000	1.0000

Heart rate dynamics.

Studies of heart rate changes in a group of animals treated with PB in drinking water for one week, preceded by one week without PB (baseline) and followed by one week of recovery after discontinuation of PB showed remarkable changes.

Table 6. ANOVA for PB effect on Heart Rate (all factors).

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A: WEEKS	2	26081.26	13040.63	9.47	0.000079*	0.980171
B: HOUR	23	430958.5	18737.33	13.61	0.000000*	1.000000
AB	46	85591.34	1860.681	1.35	0.057435	0.997184
C: DAYNUM	7	11888.84	1698.406	1.23	0.280046	0.538164
AC	14	39327.16	2809.083	2.04	0.012177*	0.960016
BC	161	1.369898E+09	8508685	6182.12	0.000000*	1.000000
ABC	322	6.231414E+07	193522.2	140.61	0.000000*	1.000000
S	3521	4846088	1376.338			
Total (Adjusted)	4096	1.223886E+07				
Total	4097					

- Term significant at alpha = 0.05

Table 7. ANOVA for PB effect on Heart Rate (day of the week excluded).

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A: HOUR	23	498830.3	21688.28	44.49	0.000000*	1.000000
B: WEEKS	2	115304.4	57652.21	118.27	0.000000*	1.000000
AB	46	13889.73	301.9507	0.62	0.974648	0.726272
S	288	140395	487.4827			
Total (Adjusted)	359	768419.5				
Total	360					

* Term significant at alpha = 0.05

Factorial ANOVA indicated significance for the factors hour of day (0-23, HOUR), weeks of experiments (week 1=baseline, week 2=PB in drinking water, week 3=recovery, WEEKS) but not for day within the week (Monday to Sunday, DAYNUM), as shown on Table 6. A second analysis was then performed by averaging data from all days of the week and redefining the model for two factors (HOUR and WEEKS). The results indicated strong effects of the two factors (Table 7).

Inspection of the graph shown in Fig. 5 indicates a wide variation of HR throughout the day, with maximal levels during the night and minimal during daylight hours. Lights were turned on at 7:00 and off at 19:00. During the week in which animals were given PB in the drinking water, significantly lower HR was observed at practically all hours. During the week following discontinuation of PB administration, HR was still significantly lower than controls, although some recovery was observed. No significant changes between groups were found in locomotor activity (Fig 6).

The analysis of HR and locomotor activity dynamics is well advanced for the control, PB and sarin treatments at 2 and 4 weeks after treatment (Tables 8-11, Figs 7 and 8). ANOVA indicated significant effects for the factors treatment, and hour of day at 2 (Tables 8-9) and 4 (Tables 10-11) weeks after treatment. HR showed lower values in the PB group at the earlier hours of the day, 2 weeks after treatment. This phenomenon may be related to a similar decrease in activity during that period of time (Fig 7). In contrast, higher activity and heart rate were observed in the PB group at 4 weeks after treatment (Fig 8).

Table 8. ANOVA for Heart Rate, 2 weeks after exposure.

Source Term	DF (Alpha=0.05)	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power
A: TREAT	2	178143	89071.51	80.38	0.000000*	1.000000
B: HOUR	23	2153212	93617.93	84.48	0.000000*	1.000000
AB	46	257627.7	5600.601	5.05	0.000000*	1.000000
S	2673	2962002	1108.119			
Total (Adjusted)	2744	5713744				
Total	2745					

* Term significant at alpha = 0.05

Table 9. ANOVA for Locomotor Activity, 2 weeks after exposure.

Source Term	DF (Alpha=0.05)	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power
A: TREAT	2	638.0982	319.0491	27.25	0.000000*	1.000000
B: HOUR	23	11651.45	506.5847	43.26	0.000000*	1.000000
AB	46	1259.083	27.37138	2.34	0.000001*	0.999999
S	2674	31312.95	11.71015			
Total (Adjusted)	2745	45980.24				
Total	2746					

* Term significant at alpha = 0.05

Table 10. ANOVA for Heart Rate, 4 weeks after exposure.

Source Term	DF (Alpha=0.05)	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power
A: TREAT	3	420225.7	140075.2	107.98	0.000000*	1.000000
B: HOUR	23	4748865	206472.4	159.17	0.000000*	1.000000
AB	69	230083.5	3334.543	2.57	0.000000*	1.000000
S	4290	5565071	1297.219			
Total (Adjusted)	4385	1.154821E+07				
Total	4386					

* Term significant at alpha = 0.05

Table 11. ANOVA for Locomotor Activity, 4 weeks after exposure.

Source Term	DF (Alpha=0.05)	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power
A: TREAT	3	5853.469	1951.156	114.63	0.000000*	1.000000
B: HOUR	23	28877.44	1255.541	73.76	0.000000*	1.000000
AB	69	4263.449	61.78911	3.63	0.000000*	1.000000
S	4265	72598.98	17.02203			
Total (Adjusted)	4360	114444.3				
Total	4361					

* Term significant at alpha = 0.05

Analysis of heart rate variability is still underway. Some preliminary results regarding RRSD and power spectrum of HR are shown in Figs 9-11. A greater variability, as estimated by RRSD, was observed for the groups with longer time after exposure to treatments, a phenomenon also observed in controls and possibly related to animal's age (Figs 9,11). Enhanced HR variability was also observed with sarin (4 weeks after treatment) and PB (4 and 16 weeks after treatment).

Power spectra indicated changes in the high frequencies (Fig 10) that were quantified by averaging power between 1 to 3 Hz (Fig 11). Findings tend to parallel those of RRSD shown in Fig 9, except that the increase in variability is notably enhanced for the dark hours (high locomotor activity) in animals that were taking PB during HR monitoring.

DISCUSSION AND CONCLUSIONS.

The results of the study on regional cerebral glucose utilization that are available at this time, indicate lack of significant changes in animals exposed to sarin under the conditions of our experimental model, at 4 weeks after treatment. Our previous findings (described in the 2002 Annual Report) had indicated a significant increase in regional cerebral blood flow of the neocortex (Fa, M2, S2, BF, FL, HL, Te, Au, Au1, V1, V2), with only a few regions showing a significant increase in Pir, RS, and Am. Regression analysis of rCBF on rCGU indicated enhancement of slope relating these two variables, a finding in line with previous results from our laboratories during acute administration of soman (Scremin et al., 1991), and carbamate cholinesterase inhibitors (Scremin et al.,

1988) (Scremin et al., 1993)at subtoxic doses. This phenomenon has been ascribed to enhancement of cholinergic transmission in neurovascular synapses (Scremin, 1991;Scremin, 1993). It is possible that residual inhibition of acetylcholinesterase in neurovascular compartments may have produced the delayed effects of sarin on cerebral blood flow reported in this experimental model. Our measurements of acetylcholinesterase activity in brain tissue, 4 weeks after discontinuation of treatment, did not reveal any significant changes (see 2002 Annual Report). However, changes in a small tissue compartment associated with cerebral blood vessels may have escaped detection given the relatively large size of samples used. Clarification of this point will require further experiments with measurements of acetylcholinesterase activity in enriched samples of cerebral microvessels.

The fact that PB treated animals had an rCBF on rCGU slope not different from normals is not surprising since this cholinesterase inhibitor does not cross the blood brain barrier. The decreased slope of animals treated with sarin + PB is difficult to explain with the information presently available. The ongoing analysis of these variables at 2 and 16 weeks post-treatment may shed light on this finding.

The functional meaning of the observed changes in CBF/CGU coupling must await the results of the analysis of electroencephalographic (EEG) activity in these same animals currently under progress. It is possible that the observed phenomenon might be associated with EEG arousal, since a similar association has been observed previously in rats (Holschneider et al., 1998;Waite et al., 1999).

The effects of treatments on cardiovascular regulation included evaluation of heart rate dynamics in the time and frequency domains. Animals were instrumented with telemetry devices and allowed to recover from anesthesia and surgical trauma for a minimum of four days to avoid interference of these factors with heart rate. The studies were conducted in the animal's home cages, thus avoiding interference by unfamiliar environments.

Heart rate showed the well known variations associated with circadian fluctuations of locomotor activity. A positive control, consisting of animals that were taking PB in the drinking water was set up, to compare results with those of animals studied after discontinuation of treatment with sarin and PB. It is well known that cholinesterase inhibitors that act peripherally, induce bradycardia in animal and human subjects (Cook et al., 2002;Nobrega et al., 2001;Serra et al., 2001;Stein et al., 1997;Nobrega et al., 1996;Yamamoto et al., 1996). Validity of our experimental model was demonstrated by significant bradycardia during PB administration.

It is important to interpret heart rate changes in relation to locomotor activity since both variables correlate positively. The significant decrease in heart rate induced by PB, both during the dark and light periods, contrasted with the lack of effects of the drug on locomotor activity. This observation is consistent with enhancement of cholinergic transmission in pre- and post-ganglionic synapses within the peripheral parasympathetic pathway innervating the sino-atrial node. The effect was still apparent, although at a

reduced magnitude, one week after discontinuation of PB treatment. A decreased heart rate was only found in the early hours of the light period, two weeks after treatment, but in this case locomotor activity, known to correlate with heart rate, was also decreased. Four weeks after treatment, heart rate was enhanced in the PB treated animals, but this change was associated, and probably caused by, enhanced locomotor activity. Previous results in restrained animals (see 2002 Annual Report) had indicated lack of effects of PB on heart rate at 2, 4 and 16 weeks after treatment. Taken together, these findings indicate a significant, although transient unbalance of autonomic influences on heart rate, limited to the period of treatment and lasting for no more than one week.

Further insight into heart rate dynamics was obtained from measurements in the frequency domain. A large increase in heart rate variability was found during administration of PB, most marked during the dark hours. This was manifested as an increase in power of the high frequency component (1-3 Hz) of the heart rate power spectrum. This phenomenon has been associated with enhanced parasympathetic tone.

Heart rate variability increased with elapsed time after exposure to treatments. This was true both when variability was measured as the average power in the high frequency range of the spectrum or as its equivalent time domain, RRSD. These increases in variability were more pronounced in those animals exposed to sarin (4 weeks after treatment) and PB (4 and 16 weeks after treatment).

The functional significance of the changes in heart rate dynamics observed cannot be fully interpreted at this time, since the database is still incomplete and these results should only be considered as preliminary. It is however noteworthy that, as expected, heart rate dynamics appears to be a sensitive index of alterations in parasympathetic tone induced by cholinesterase inhibition. Depression of heart rate variability has been associated with autonomic neuropathy, congestive heart failure, myocardial infarction and many other diseases (Stys and Stys, 1998), and it has been ascribed prognostic value in the prediction of arrhythmias and sudden death after myocardial infarction (Kleiger et al., 1987). Heart rate variability has also been used to follow up the course of diabetic neuropathy. PB is known to enhance heart rate variability in humans (Nobrega, dos Reis, Moraes, Bastos, Ferlin, and Ribeiro, 2001), and this drug has been advocated for clinical use intending to improve the outcome of myocardial infarction (Castro et al., 2002). Thus, the enhanced heart rate variability observed in our experimental model that intends to mimick conditions of administration of PB as a prophylactic of nerve agent intoxication, may not be considered adverse. The same reasoning may apply to the enhanced heart rate variability observed with sarin after the three intervals of time following treatment.

KEY RESEARCH ACCOMPLISHMENTS.

- PB administration induced significant bradycardia and enhancement of heart rate variability, without significant changes in locomotor activity. These changes persisted for one week after discontinuation of treatment.
- Enhancement of heart rate variability as estimated by time (RRSD) and frequency (power spectrum) domain measurements was observed with sarin (4 weeks after treatment) and PB (4 and 16 weeks after treatment).
- Glucose utilization of the cerebral cortex did not show significant changes between treatments, 4 weeks after exposure to drugs.
- The ratio of cerebral blood flow to cerebral glucose utilization was found to be enhanced by sarin and depressed by the combination of sarin and PB, 4 weeks after treatments.

REPORTABLE OUTCOMES.

Scremin, O.U., T.M. Shih, Huynh,L., Roch, M., Booth, R, and Jenden, D.J. Delayed Neurologic And Behavioral Effects Of Sub-Toxic Doses Of Cholinesterase Inhibitors.
Published November 25, 2002. 10.1124/*J. Pharmacol. Exp. Ther.*102.044818.

Scremin, O.U., Shih, T.M. , Huynh,L., Roch, M., Booth, R., D'Elia, J., Cable, C. Sreedharan, A., and Jenden, D.J. Effects of Low-Dose Cholinesterase Inhibitors on Cognition. *Med. Defense Biosci. Rev. 2002*, DTIC.

Scremin, O.U., Shih, T.M. , Huynh,L., Roch, M., Booth, R., D'Elia, J., Cable, C. Sreedharan, A., and Jenden, D.J. Pyridostigmine Bromide Prevents Delayed Neurological Effects of Low Dose Sarin. *Med. Defense Biosci. Rev. 2002*, DTIC.

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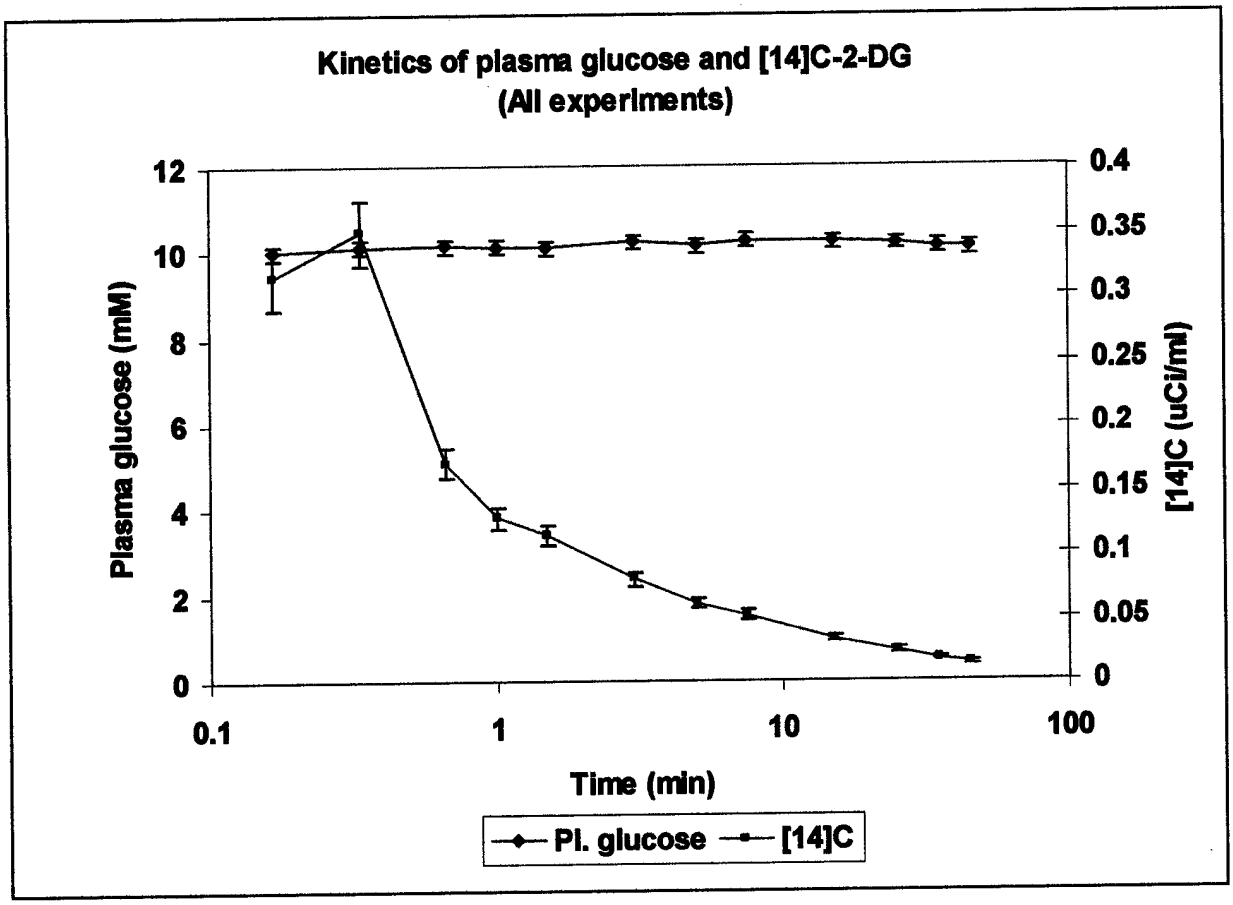


Figure 1: Kinetics of plasma glucose and plasma in experiments in which cerebral glucose utilization was measured. Data are means and SE of all experiments. The level of plasma glucose was uniform throughout the 45 min experiment, and ^{14}C-2-DG, injected as a rapid infusion over 30 seconds followed the expected decay with time after infusion of the tracer ceased. ANOVA of plasma glucose levels showed no significance for the factors treatment (Control, Sarin, PB and Sarin+PB), time after treatment (2, 4, and 16 weeks), or their interaction.

Cortical rCGU maps
4 weeks after treatment

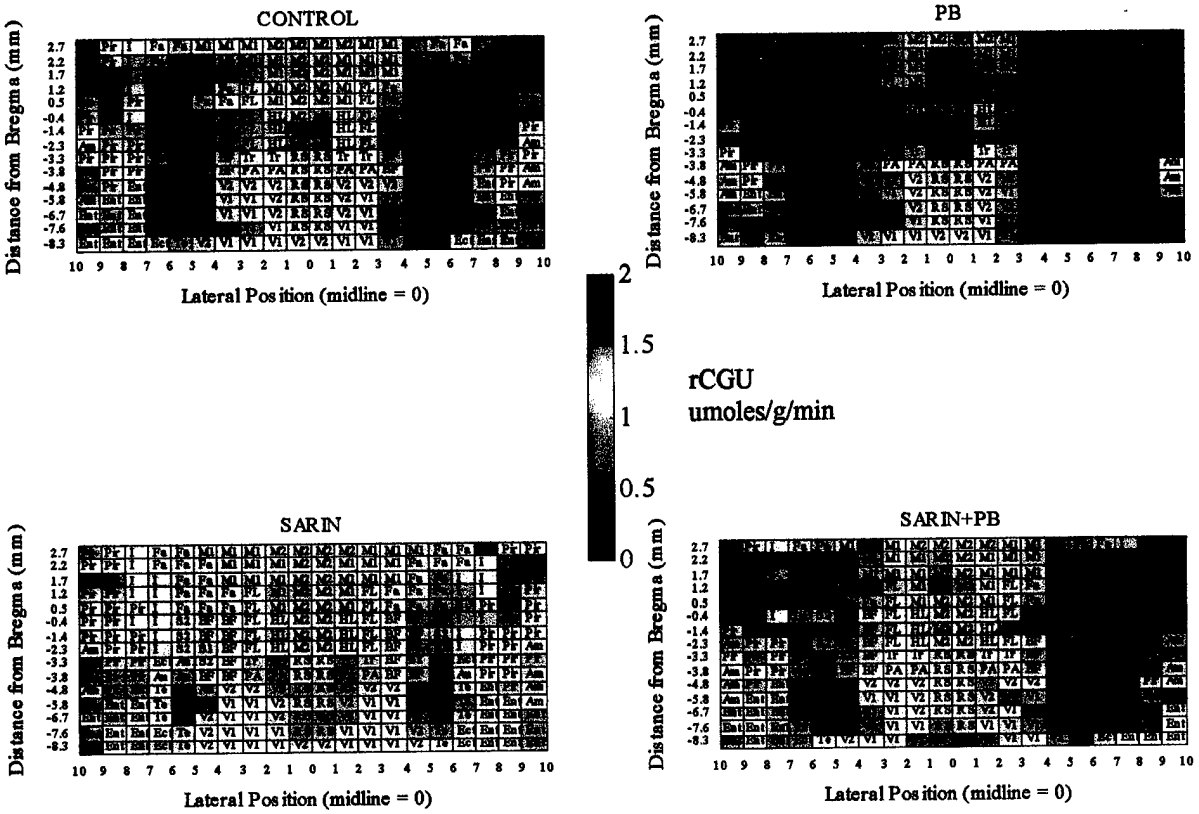
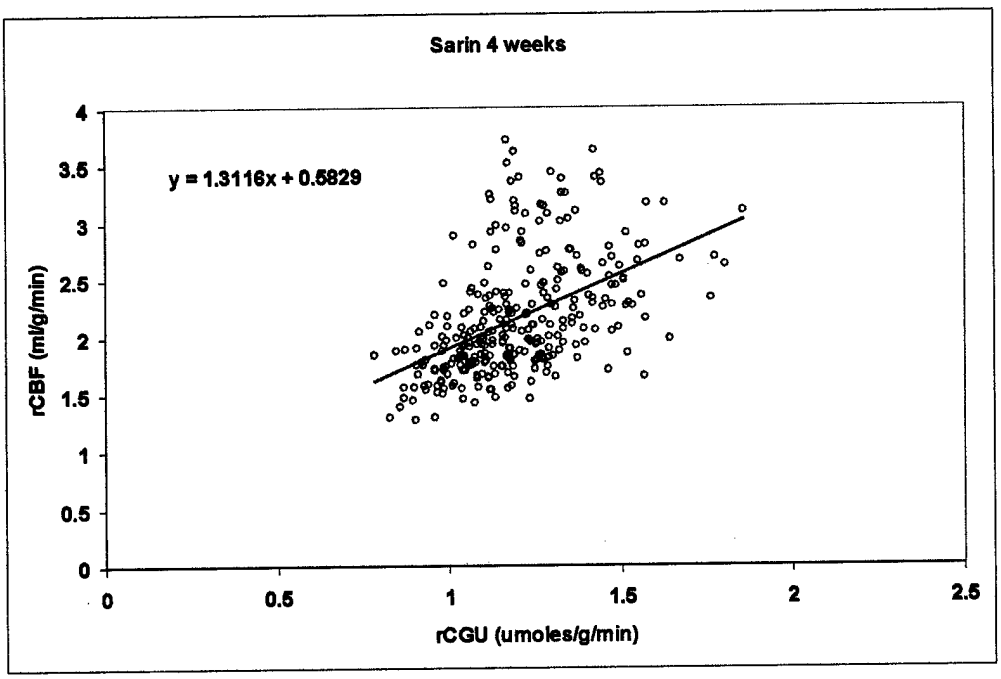


Figure 2: Cerebral glucose utilization (rCGU) was measured with the ^{14}C-2DG technique in 9 conscious rats per group, 4 weeks after treatment. 300 cortical regions were sampled in 15 coronal planes, identified by their distance from bregma in mm (ordinate), and their relative position from the midline (abscissa). Negative plane values are caudal and positive plane values are rostral to bregma. Regions are named according to the Atlas of Paxinos and Watson (Academic Press, San Diego, 4th Edition, 1998). See text for abbreviations. None of the regional differences between treatments and controls were statistically significant.



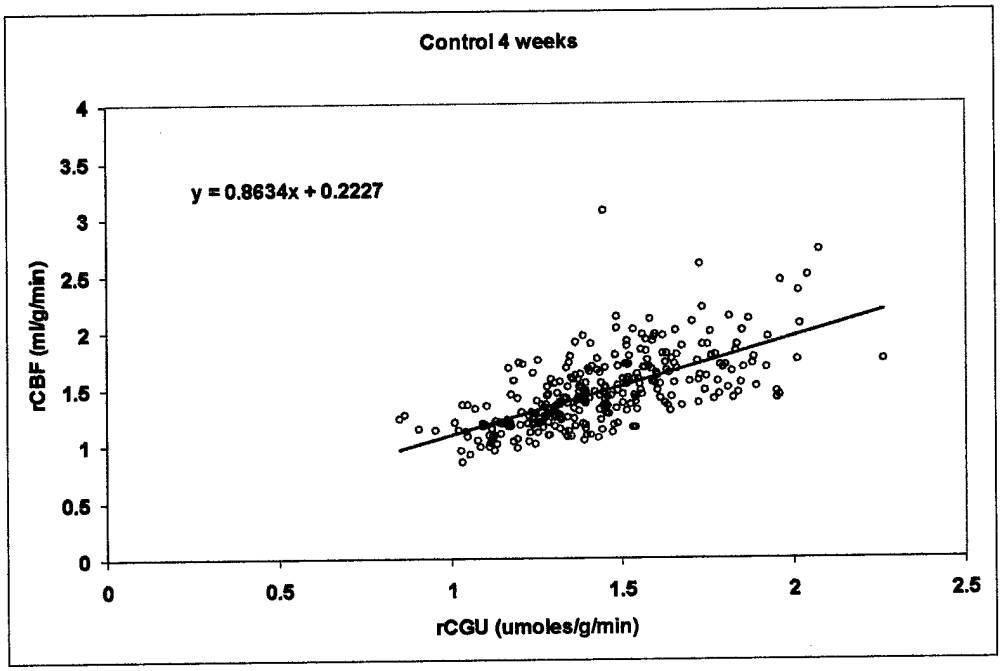
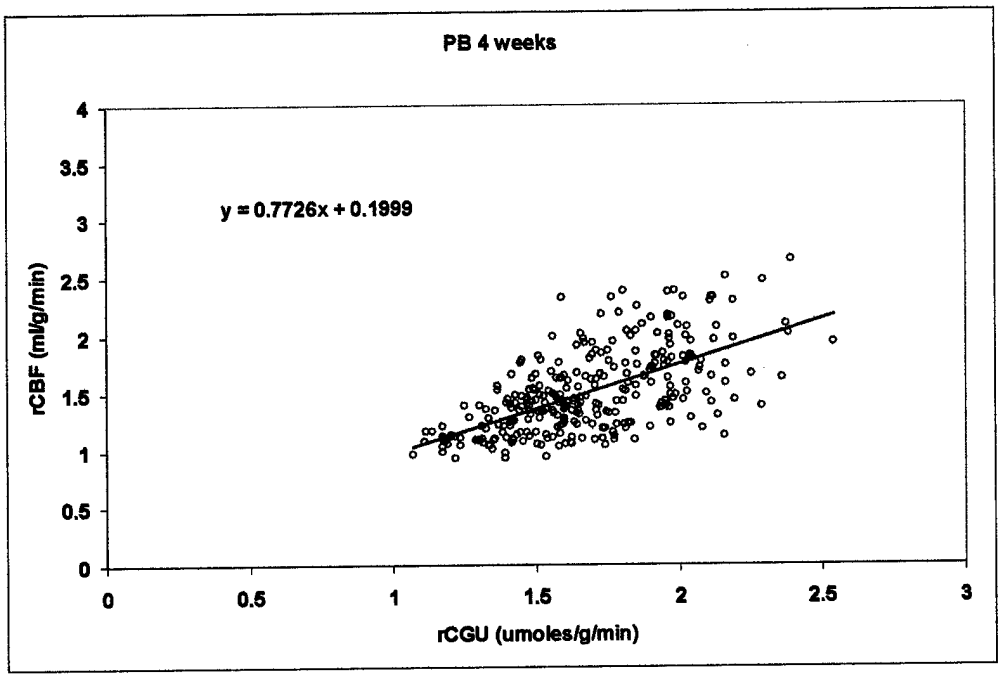


Figure 3: Cerebral glucose utilization (rCGU) was measured with the ^{14}C-2DG technique in 9 conscious rats per group, and cerebral blood flow (rCBF) with the ^{14}C- IAP technique in 12 rats per group, 4 weeks after treatment. 300 cortical regions were sampled in 15 coronal planes. Paired values of rCBF and rCGU are plooted. The line represents the slope of the regression of rCBFon rCGU. ANOVA of regression indicated that both regression coefficients were different from zero. The slope of the sarin group was significantly higher thatn the slope of the control group.See text for significance tests.



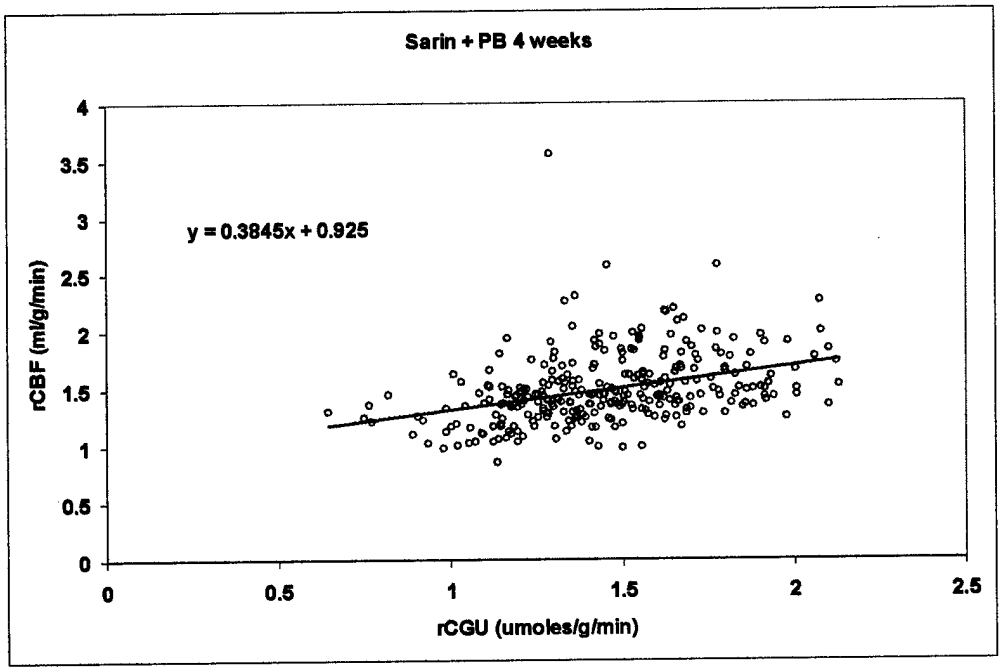


Figure 4: Cerebral glucose utilization (rCGU) was measured with the ^{14}C-2DG technique in 9 rats per group, and cerebral blood flow (rCBF) with the ^{14}C- IAP technique in 12 rats per group, 4 weeks after treatment. 300 cortical regions were sampled in 15 coronal planes. Paired values of rCBF and rCGU are plooted. The line represents the slope of the regression of rCBFon rCGU. ANOVA of regression indicated that both regression coefficients were different from zero. The slope of the sarin + PB group was significantly lower than the slope of the control group. See text for significance tests.

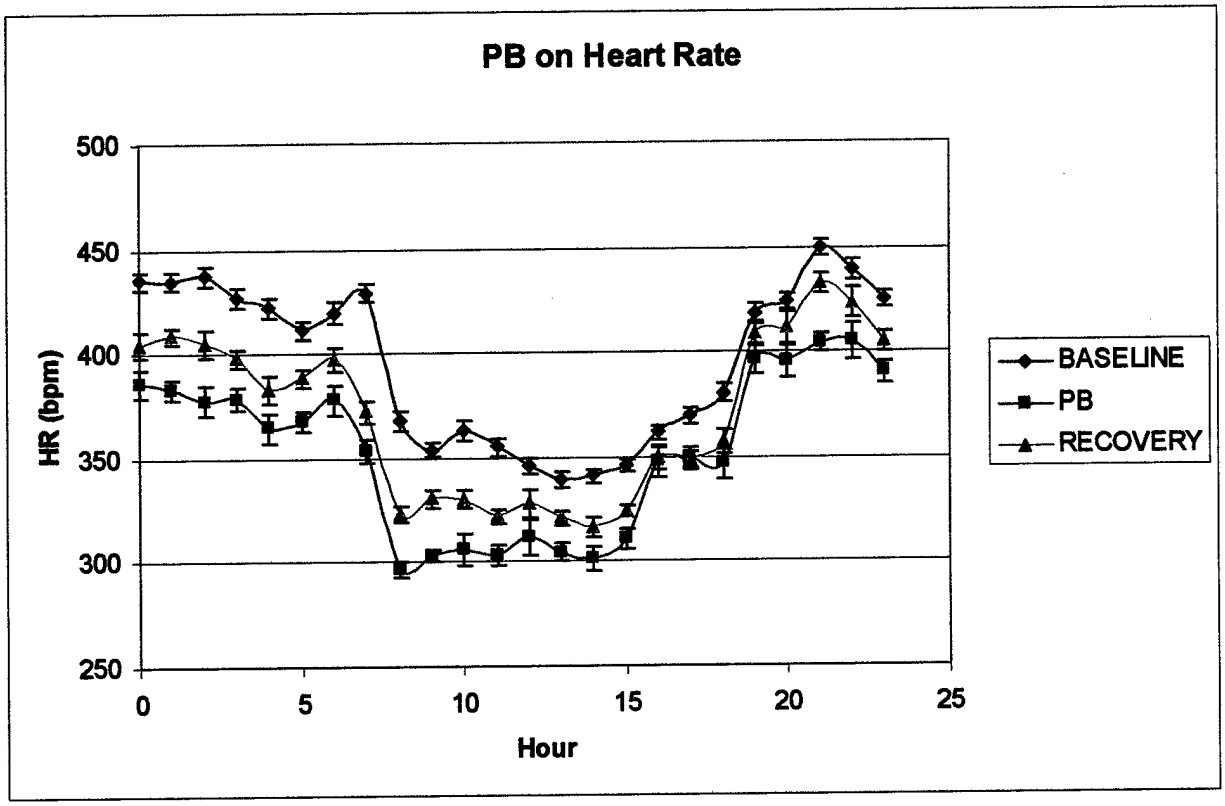


Figure 5: Heart rate (HR) was measured in five rats from the ECG recorded with previously implanted radiotelemetry transmitters while the animals were in their home cages, 24 hrs a day, every hour, during seven days. Lights were turned on at 07:00 and off at 19:00. Data shown is the average of seven days of recording for all animals in each experimental condition. ANOVA (see text Table 2) indicated significance for the factors weeks (baseline, PB, recovery), and hour of day (indicated in the abscissa). Individual contrasts among means of the three experimental conditions (Fisher LSD) indicated that PB HR was different from baseline HR at all hours of day, except 16, 20, 21, and 22. HR during the week after PB was discontinued (recovery) was significantly different from control at hours 4, 7, 8, 10, 11, 14, 15, and 17.

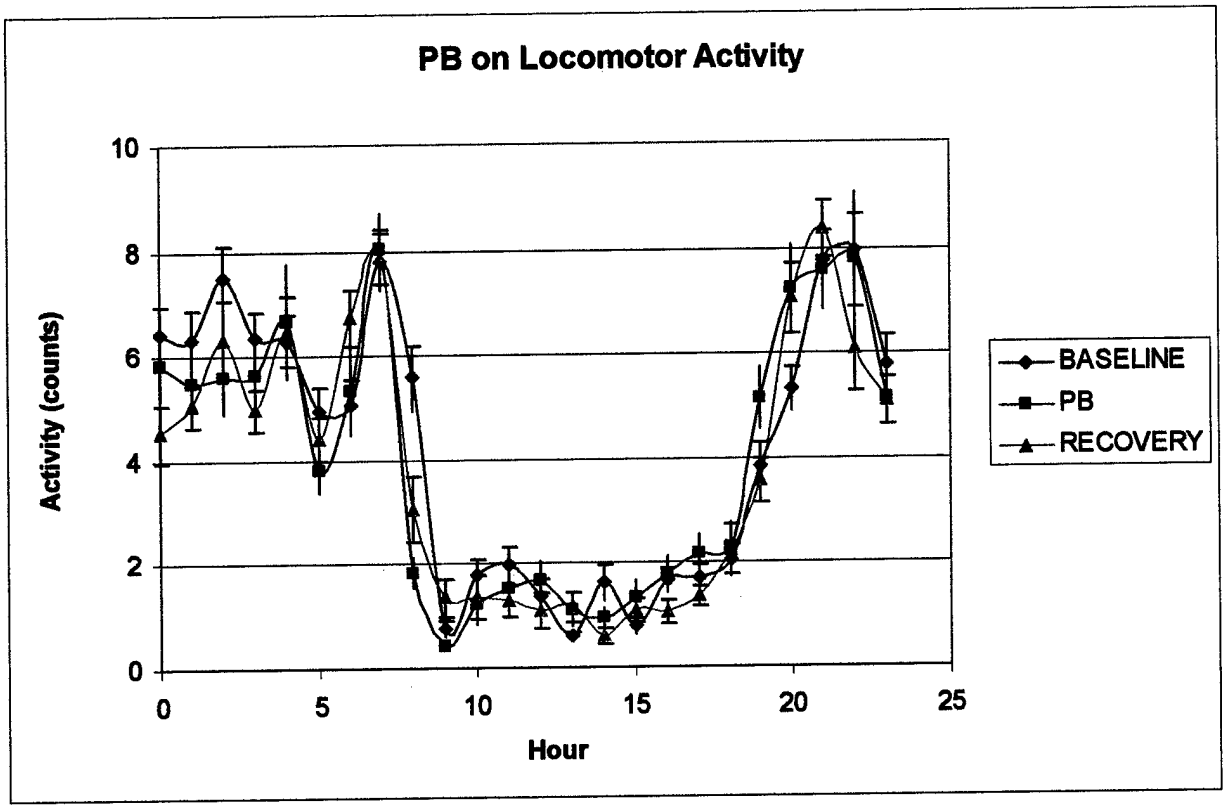


Figure 6: Locomotor activity was measured in five rats with previously implanted radiotelemetry transmitters while the animals were in their home cages, 24 hrs a day, every hour, during seven days. Lights were turned on at 07:00 and off at 19:00. Data shown is the average of seven days of recording for all animals in each experimental condition. There were no significant differences between activity values of weeks two (PB) and three (recovery) and those of week one (baseline.

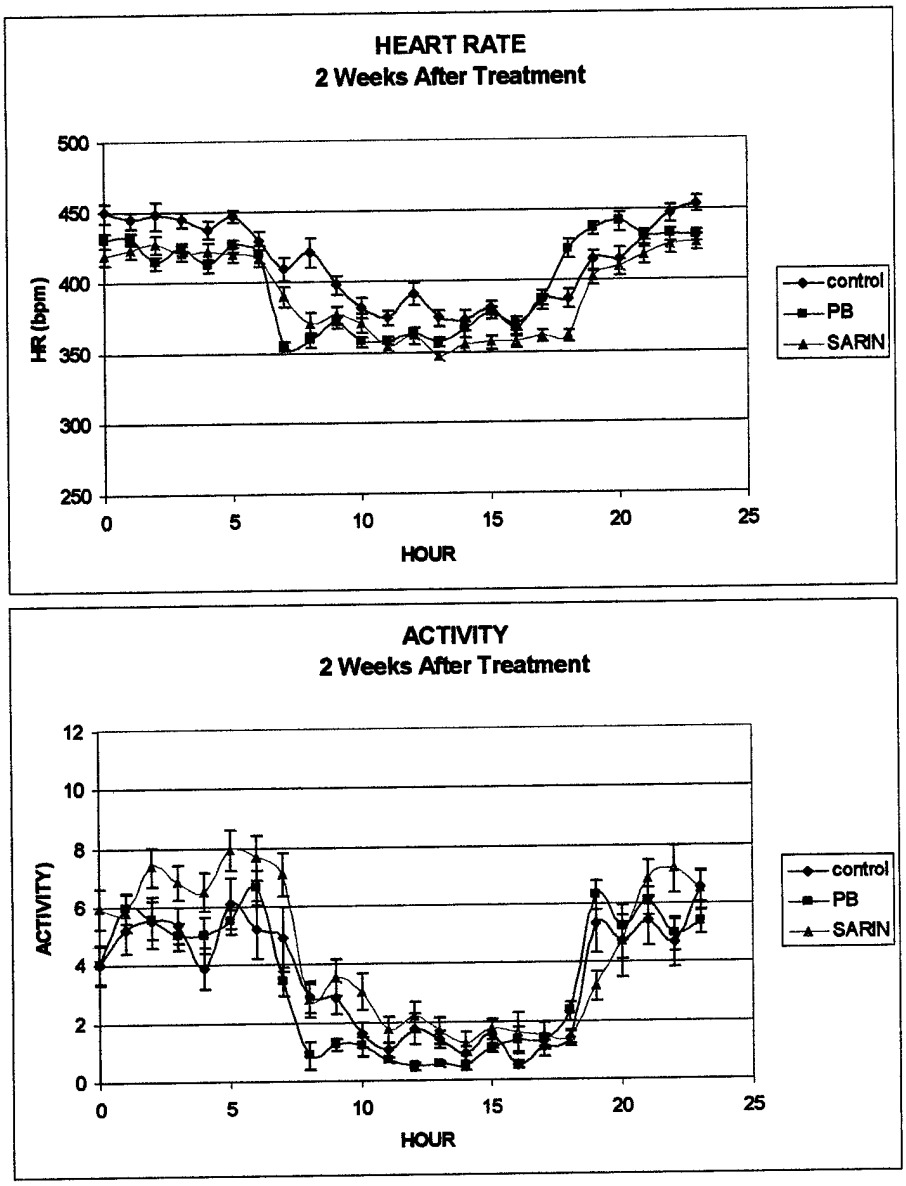
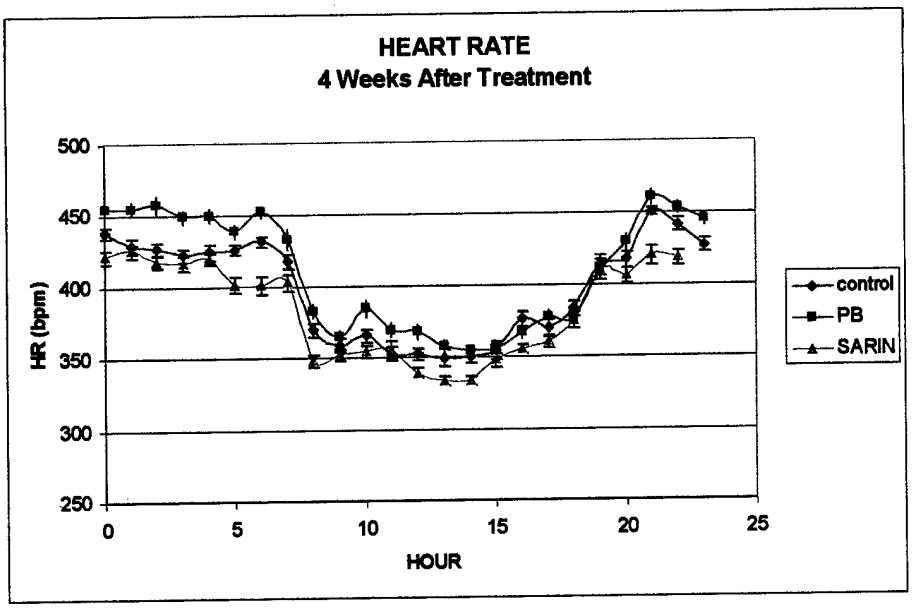


Figure 7: Heart rate and locomotor activity, 2 weeks after treatment, were measured with previously implanted radiotelemetry transmitters while the animals were in their home cages, 24 hrs a day, every hour, during seven days. Lights were turned on at 07:00 and off at 19:00. Data shown are averages of seven days of recording for all animals in each experimental condition. ANOVA (see text Tables 3-4) indicated significance for the factors hour of day and treatment.



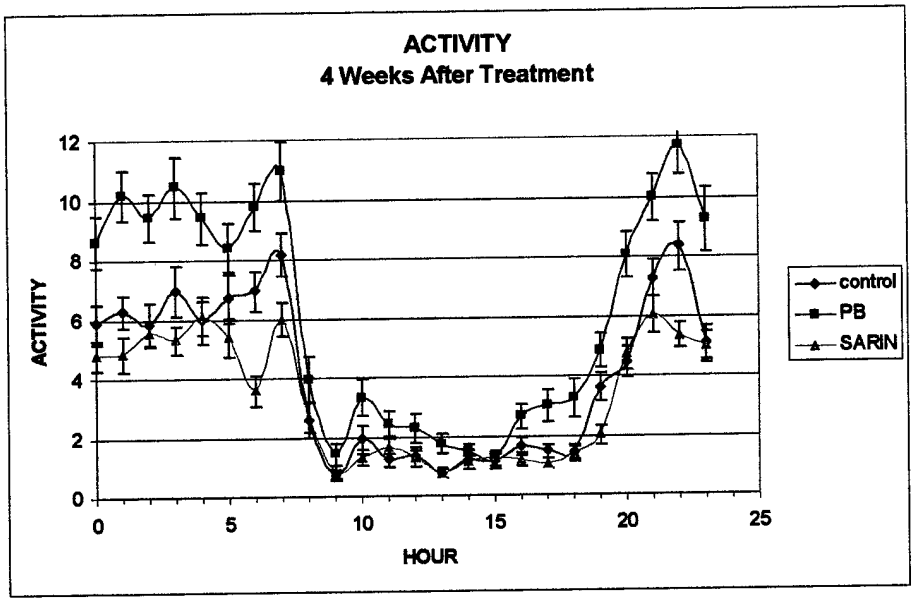


Figure 8: Heart rate and locomotor activity, 4 weeks after treatment, were measured with previously implanted radiotelemetry transmitters while the animals were in their home cages, 24 hrs a day, every hour, during seven days. Lights were turned on at 07:00 and off at 19:00. Data shown are averages of seven days of recording for all animals in each experimental condition. ANOVA (see text Tables 3-4) indicated significance for the factors hour of day and treatment.

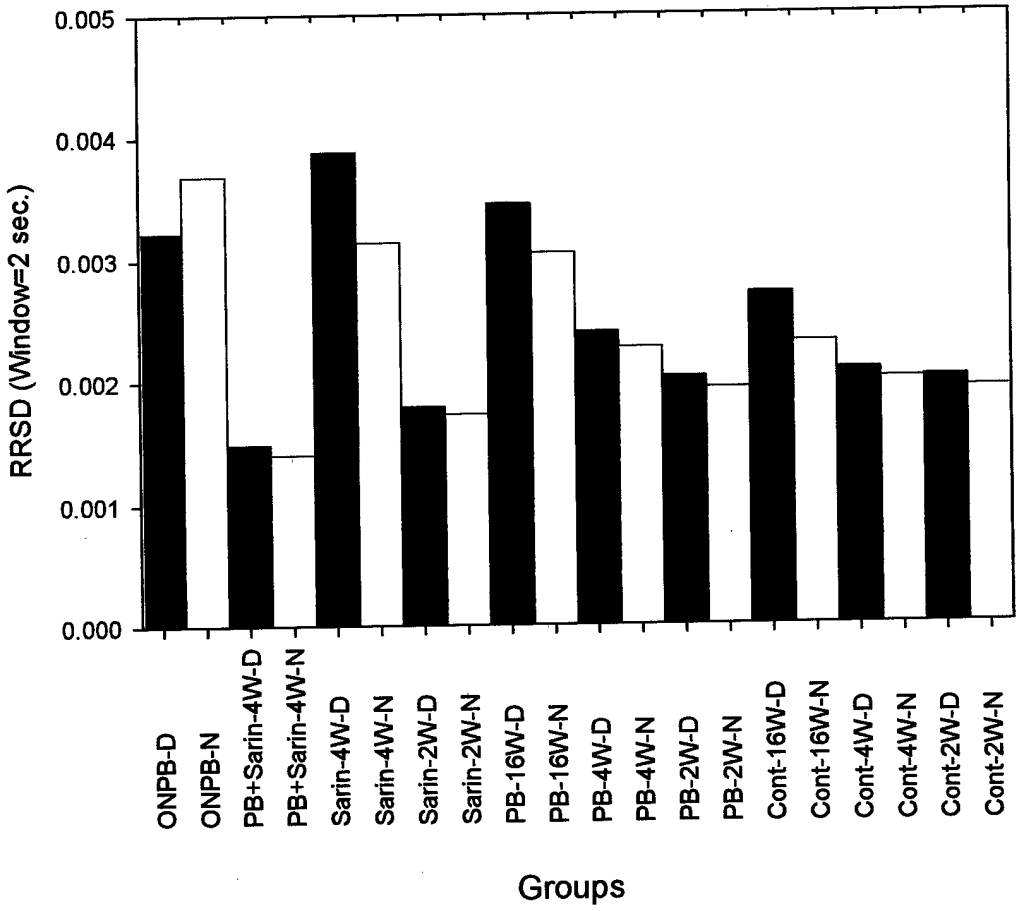


Figure 9: Mean standard deviation of RR intervals are plotted for the groups indicated in the abscissa. D= light hours (07:00 to 19:00); N= dark hours (19:00 to 07:00); ONPB= animals taking PB in drinking water during one week and monitoring during the week of treatment. All other groups were monitored at 2 (2W), 4 (4W), or 16 (16W) after treatment was discontinued. Control= drinking tap water and given saline injections; PB= treated with PB in drinking water during three weeks; Sarin= treated with Sarin s.c. during three weeks; PB+Sarin= taking PB in drinking water and given Sarin s.c. during three weeks.

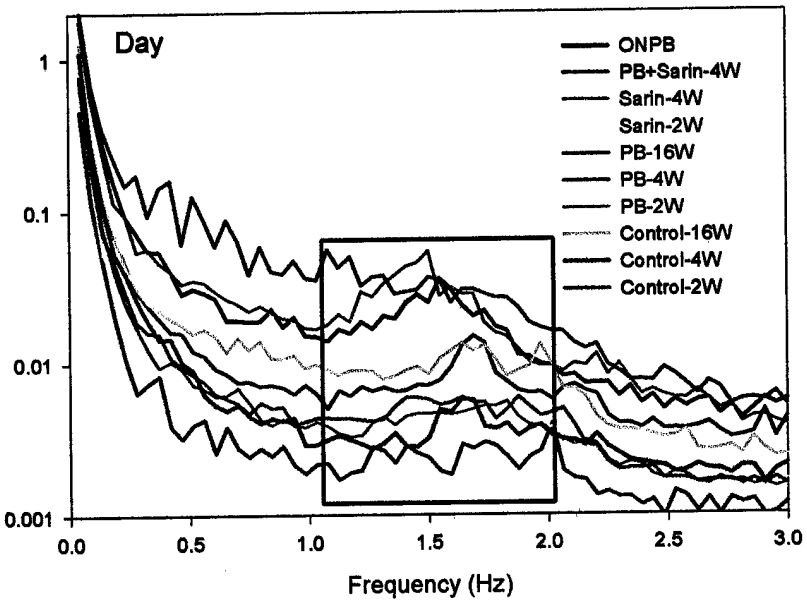


Figure 10: Power spectra shown were calculated from light hours records (day) using a Fast Fourier Transform after re-sampling the time series of RR intervals at a rate of 6 Hz. for the groups indicated in the insert. ONPB= animals taking PB in drinking water during one week and monitoring during the week of treatment. All other groups were monitored at 2 (2W), 4 (4W), or 16 (16W) after treatment was discontinued. Control= drinking tap water and given saline injections; PB= treated with PB in drinking water during three weeks; Sarin= treated with Sarin s.c. during three weeks; PB+Sarin= taking PB in drinking water and given Sarin s.c. during three weeks. The rectangle around 1.5 Hz indicates the location of the "high frequency" peak, thought to correlate with cholinergic tone.

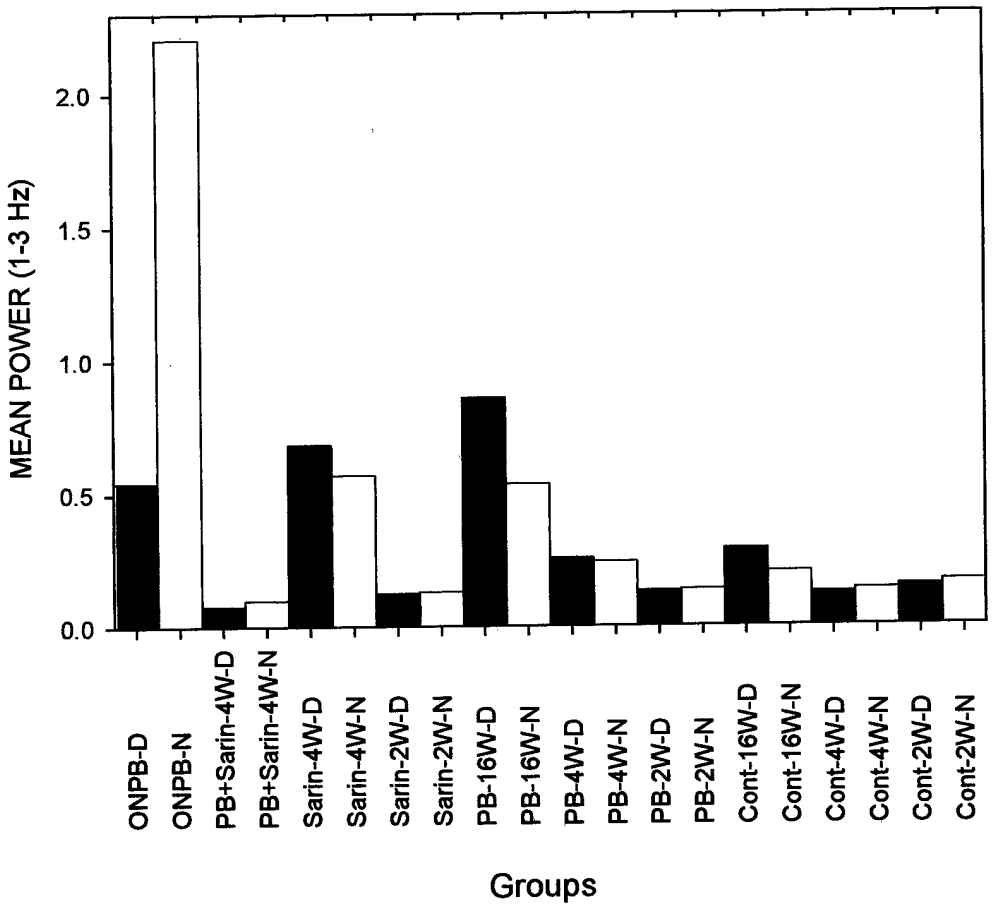


Figure 11: The mean cumulative power in the range of 1 to 3 Hz was calculated from each data set and averaged for the groups indicated in the abscissa. D= light hours (07:00 to 19:00); N= dark hours (19:00 to 07:00); ONPB= animals taking PB in drinking water during one week and monitoring during the week of treatment. All other groups were monitored at 2 (2W), 4 (4W), or 16 (16W) after treatment was discontinued. Control= drinking tap water and given saline injections; PB= treated with PB in drinking water during three weeks; Sarin= treated with Sarin s.c. during three weeks; PB+Sarin= taking PB in drinking water and given Sarin s.c. during three weeks.

APPENDICES

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JPET/2002/44818

DELAYED NEUROLOGIC AND BEHAVIORAL EFFECTS OF SUB-TOXIC DOSES OF CHOLINESTERASE INHIBITORS.

Oscar U. Scremin, Tsung-Ming Shih, Ly Huynh, Margareth Roch, Ruth Booth, and Donald J. Jenden.

Department of Research, VA GLA Healthcare System (O.U.S., L.H., M.R., R.B.), U.S. Army Medical Research Institute of Chemical Defense (T.-M.S.), Departments of Physiology (O.U.S.) and Medical and Molecular Pharmacology (D.J.J.), UCLA School of Medicine.

RUNNING TITLE: DELAYED EFFECTS OF LOW-LEVEL SARIN AND PYRIDOSTIGMINE BROMIDE.

Corresponding Author:

Oscar U. Scremin, MD, PhD
Department of Research, VA GLA Healthcare System
11301 Wilshire Blvd, Bldg 115, Rm 319
Los Angeles, CA 90073
Phone: (310) 268 3895; Fax: (310) 268 4209
Email: oscremin@ucla.edu

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Discussion, 1226 words

Abbreviations:

AChE= acetylcholinesterase; BuChE= butyrylcholinesterase; ChAT= cholineacetyltransferase; PB= pyridostigmine bromide.

ABSTRACT

We tested the hypothesis that pyridostigmine bromide (PB) intake and/or low level sarin exposure, suggested by some as causes of the symptoms experienced by Persian Gulf War veterans, induce neurobehavioral dysfunction that outlasts their effects on cholinesterase. Adult male Sprague-Dawley rats were treated during three weeks with subcutaneous (s.c.) saline, PB in drinking water (80 mg/L), sarin (62.5 μg/kg; 0.5xLD50, three times/week s.c.), or PB in drinking water + sarin. Animals were tested for passive avoidance, nociceptive threshold, acoustic startle, and open field activity 2, 4 or 16 weeks after treatment.

Two weeks after sarin, acoustic startle was enhanced while distance explored in the open field decreased. These effects were absent with PB + sarin or PB by itself. No effect on any variable was found at 4 weeks, while at 16 weeks sarin induced a decrease and PB + sarin an increase in habituation in the open field test. Nociceptive threshold was elevated in the PB + sarin group at 16 weeks. No effect of treatment on passive avoidance was noted in any group. Brain regional AChE and ChAT activities were not affected at any time after treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon in the sarin group at 2 weeks.

In conclusion, this study gives further support to the use of PB against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level sarin exposure.

Many veterans of the Persian Gulf War complain from clusters of symptoms including cognitive alterations, balance disturbances and vertigo, and muscle aches and weaknesses (Haley, 2001), which have been ascribed by some authors, among other possible factors, to exposure to the ChE inhibitors pyridostigmine bromide (PB), a carbamate, and/or sarin, a highly toxic organophosphorus (OP) chemical warfare nerve agent.

PB, like other carbamate ChE inhibitors, protects animals from the lethal effect of OP ChE inhibitors when given in anticipation of exposure to these OP agents. The mechanism of this protection appears to be the pre-occupation by the carbamate of ChE reactive sites, which become unavailable to the OP ChE inhibitor, with subsequent restoration of enzymatic activity due to the reversible decarbamylation of ChE. This phenomenon is the basis for the use of PB as a prophylactic of nerve agent intoxication (Dirnhuber et al., 1979; Leadbeater et al., 1985; Koplovitz et al., 1992; Kluwe et al., 1987; Keeler et al., 1991). The therapeutic target for this application of PB has been to maintain inhibition of plasma butyrylcholinesterase (BuChE) between 20% to 40%. Large scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler et al., 1991). Possible exposure to sarin may have occurred following explosions of ammunition dumps with consequent air contamination at Khamisiyah, Iraq (McCauley et al., 2001).

The effects of low-level repeated exposure to OP nerve agents, not associated with acute clinical signs or symptoms, have attracted less attention than the well known effects of acute intoxication with these agents (Ecobichon and Joy, 1982; Sidell, 1974; Chambers, 1992) . Behavioral and electroencephalographic alterations in workers exposed to low levels (not associated with acute intoxication) of nerve agents have been reported (Burchfield and Duffy, 1982; Ecobichon and Joy, 1982). However, a study of human volunteers exposed to low to moderate levels of nerve agents has indicated no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer (Panel on Anticholinesterase Chemicals, 1982; Coordinating Subcommittee, 1985). The same conclusion appears to hold for low-level accidental exposures to OP nerve agents (Moore, 1998).

The present study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf War veterans, could elicit cognitive or neurobehavioral abnormalities in experimental animals. Our initial experiments were aimed at establishing the optimal doses of sarin and PB. For sarin, the optimal dose was defined as the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment. This criterion was adopted because no episodes compatible with symptoms of acute intoxication with ChE inhibitors have been described in soldiers during the Persian Gulf War, although it is possible that low-level exposure to sarin may have occurred. In the case of PB, the optimal dose was defined as one producing 20-30% inhibition of plasma BuChE. This is the degree of BuChE inhibition reported for human subjects receiving the same PB

dosage as soldiers during the Persian Gulf War (Keeler et al., 1991) (90 mg PB over 24 hrs, divided in three oral doses).

Passive avoidance and open field activity tests were used to assess cognitive function and motor activity, respectively. Auditory startle and nociceptive threshold were assessed to determine the existence of possible neurological dysfunction. In addition, we analyzed, in key brain regions, the activity of ChAT and AChE, the enzymes responsible for ACh synthesis and degradation, respectively, as well as the expression of muscarinic cholinergic receptors in the same animals that were subjected to the neurobehavioral tests mentioned above. Separate groups of animals were studied at 2, 4, or 16 weeks after 3 weeks of exposure.

METHODS.

Male Crl:CD(SD)IGSBR Sprague-Dawley rats, weighing 250-300g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12- h light-dark full spectrum lighting cycle with lights on at 0600 h. Laboratory chow and water were freely available. Experiments were conducted at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml/kg subcutaneously (s.c.). PB was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a three-week period.

Determination of optimal doses of sarin, PB and their combination: A preliminary verification of the LD50 of sarin in rats was conducted by the "up and down" method (Dixon, 1965) using 5 doses (3 animals per dose level) with 120 μg/kg as the middle dose at intervals of 0.05 Log_{10} unit. To find the optimal dose for sarin, animals were administered LD50 doses of this agent in 0.1 unit increments starting from 0.2 and

up to 0.7xLD50, three times (Mondays, Wednesdays, and Fridays) per week for three weeks in groups of 6 animals per dose. The highest dose not associated with toxic signs (described in detail below) during this 3-week period was adopted for the main study.

After correction for surface area equivalence between rats and human subjects (Freireich et al., 1966), the PB rat dose equivalent to that used in humans during the Persian Gulf War was calculated as 9 mg/kg/day. Experiments were set up to measure the plasma butyrylcholinesterase (BuChE) activity as well as the possible existence of signs of cholinergic toxicity in animals receiving 2.5, 5, 10 or 20 mg/kg/day PB in the drinking water during three weeks. Prior to this, the average daily drinking volume for the set of rats to be used (as ml of water intake per kg body mass per day) was determined by measuring volume of water consumption over a three-week period. This pilot study indicated that to achieve the desired daily doses described above, animals should be given PB in the drinking water at concentrations of 20, 40, 80, and 160 mg/L, respectively. The effects of PB treatment on plasma BuChE were monitored.

The optimal repeated dose of sarin to be used in combination with PB in drinking water at a concentration determined by the previous study was established as follows. While taking PB in drinking water, animals were administered doses of 0.3, 0.4, 0.5 or 0.6 LD50 sarin s.c., three times (Mondays, Wednesdays, and Fridays) a week for three weeks in groups of 6 animals per dose.

Experimental groups: Separate sets of animals were studied at 2, 4, or 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Group 1

served as overall control. These animals received regular tap water as drinking water and were injected with saline (Control group). Group 2 animals received PB in drinking water (80 mg/L) and were injected with saline (PB group). Group 3 animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to 0.5xLD50) (Sarin group). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above (PB + sarin group). PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 08:00 hour. At 09:00 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 17:00 hour on Friday of the third week. Animal dosing procedures were performed at the USAMRICD laboratory. After a period of 1, 3, or 15 weeks following treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-freight to the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables at 2, 4, or 16 weeks after control, PB, sarin, or PB + sarin treatments. Telemetry measurements of locomotor activity and heart rate performed in animals after they arrived at the VA Greater Los Angeles Healthcare System (data not shown), have indicated normal circadian rhythms in animals transported under the same conditions and studied at the intervals used in the present report. Moreover, in this experimental design all animals (treated and controls) were transported in the same way in order to cancel out any potential differences due to transportation stress.

Number of animals was 12 per group, and the total number of groups (treatments x times after treatments) was also 12 with a grand total of 144 rats.

Observation of signs of intoxication: Animals were observed for signs of cholinergic intoxication for at least one hour following sarin injection. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described elsewhere (Shih and Romano, 1988).

Blood ChE measurements: When animals were received at the USAMRICD laboratory, they were allowed to acclimate for a week. During this period blood was collected from the tail vein (Liu et al., 1999) on two separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period.

Blood was collected into an Eppendorf 1.5 mL microtube containing 50 µL (1000 USP unit per ml) heparin sodium and mixed. Forty µL of whole blood were transferred to another microtube containing 160 µL 1% Triton-X 100 (in saline) solution, mixed well and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 RPM (20,000 RCF). Plasma was carefully aspirated off, and 20 µL RBC's was

transferred into a microtube containing 180 µL 1% Triton-X 100 solution. The tube was tapped firmly until RBC's were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at –75°C until ChE analysis. At the time of analysis, samples were processed immediately after thawing to avoid spontaneous re-activation or additional inhibition of ChE activity. Whole blood and RBC AChE activity were determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics Inc., Nutley, NJ). The analytical procedure was based on the manual method of Ellman (Ellman et al., 1961) and modified for the COBAS/FARA system using acetylthiocholine as substrate. Plasma butyrylcholine activity was measured with the same method, but by using butyrylthiocholine as substrate, and manual readings of kinetic data on a Beckman scanning spectrophotometer.

Regional brain activity of ChAT and AChE, and QNB binding: Animals were euthanized by decapitation while under deep halothane anesthesia (2.5% in 30% O_2 balanced with N_2O). The brain was rapidly removed and flash frozen in methylbutane cooled to – 70 °C. Brain regions were microdissected from frozen brain slices for the following ten anatomical locations in each animal: somato-sensory, temporal, and pyriform cortex, hippocampus, caudate-putamen, thalamus, hypothalamus, mesencephalon, cerebellum, and medulla. These tissue samples were homogenized, and aliquots of these homogenates were used to determine tissue AChE activity with the kinetic method of Ellman (Ellman et al., 1961), ChAT activity with the method of

Fonnum (Fonnum, 1975), and quinuclydinyl benzilate (QNB) binding with saturation assays (Yamamura and Snyder, 1974).

Inhibited (passive) avoidance response: This was measured in a "step through" apparatus (McGaugh, 1972), consisting of (a) a small compartment made of white plastic, (b) a larger, dark compartment of stainless steel, and (c) a shock delivery unit adjustable for the intensity and duration (1 mA, 0.5 sec) of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 hrs. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment lead immediately to the closing of a door and administration of footshock. Retention was tested after a 48-hr delay, the measure being time taken to enter the dark compartment after release from the white compartment. The time to enter was defined as "retention," a measure of memory of the single training session. The retention trials were set at a limit of 10 min.

Open field locomotor activity: This was measured during a 20-min session in circular open field chambers of 60 cm diameter, with walls 45 cm high, under low-level red light illumination. This is done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a Sony CCD video camera (sensitive to the wavelength of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc., The Netherlands). Tracking was

performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Distance traveled was summated at 1-min intervals, and these values were fitted by non-linear regression, using the Marquardt algorithm, to the model:

$$Y = A \cdot e^{-Bt} \quad (1)$$

where Y = distance moved (cm) and t= time after initiation of test (min). The values of parameters A (initial velocity, cm min^{-1}) and B (habituation, min^{-1}) were obtained as described above for every animal. Analysis of variance (ANOVA) was then performed for the two parameters using the factor treatment (control, PB, sarin and PB + sarin) at every time after treatment (2, 4, or 16 weeks).

In addition, total distance traveled and mean distance to the arena's border (the inner surface of the chamber's wall) during the entire test were also calculated for every animal.

Reactivity (startle response): Reactivity is defined as a response to a sudden brief and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been previously described (Silverman et al., 1988; Russell and Macri, 1979). In this procedure the animals stand unrestrained on a platform provided with a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom-designed computer program delivers a controlled sound and integrates and digitizes the movement-related

electrical signal. Quantification of the response is provided in arbitrary force units. In the currently reported experiments, 20 trials were performed at fixed intervals of 10 seconds.

Nociceptive threshold: The procedure to measure nociceptive threshold used in these experiments has been described (Crocker and Russell, 1984) and utilizes reaction to a mild electric foot shock as its measure. It involves the "up and down" method (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 sec at 10-sec intervals. The shock intensities were available in a range from 0.05 mA to 0.4 mA and arranged in a $\log_{10}$ scale at 0.1 $\log_{10}$ units. Shock levels were set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animal's response on each trial. A "flinch" was defined as an elevation of 1 or 2 paws from the grid floor and "jump" as rapid withdrawal of three or more paws from the grid.

Data Analysis: Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data is presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) at each time after exposure to drugs or saline with one factor (treatment) at four levels (control, PB, sarin, PB + sarin). This was followed, if significant (probability

for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method.

RESULTS.

Dose Finding Studies: The LD50 of sarin was determined to be 125 μg/kg, sc. An initial evaluation indicated that animals whose drinking water contained PB at a concentration of 80 mg/L had inhibition of plasma BuChE slightly greater than 20% on average. This was within the target effect set for these experiments (20 to 30% inhibition). The next higher PB concentration in drinking water (160 mg/L) induced a larger plasma BuChE inhibition (between 27 and 40 %). Thus, the concentration of 80 mg/L PB in drinking water was adopted for the rest of the study. No sign of toxicity, as defined in Methods, was found in animals drinking water containing PB during three weeks.

The dose finding for sarin and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose that did not cause observed acute toxic effects when given alone or in combination with PB (80 mg/L in drinking water) for a period of three weeks.

Body mass: Means of body mass, recorded daily during weekdays, through the three weeks of treatment and the subsequent two weeks following treatment are shown in Fig 1. No statistically significant difference was found between treatments. The expected increase in body mass with age was observed at the beginning of the experiments that assessed outcome variables (2, 4 or 16 weeks after treatment), but no difference among treatment groups was found at these time points either.

Blood ChE activity: Measurements of RBC AChE during the 3 drug treatment weeks, the pre-treatment week (two measurements) and 3 post-treatment weeks are shown in Fig 2. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the three weeks of treatment. Sarin and PB + sarin produced an average decrease in RBC AChE to 35% and 27% of pre-treatment respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

Nociceptive threshold : Data are presented in Fig. 3 for both the flinch and jump responses.

Flinch response: No statistically significant difference among groups was found for the flinch response to the test at 2 or 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher LSD test, $P<0.05$) showed that the nociceptive threshold of the animals that received the combination of PB + sarin (0.117 ± 0.011 mA) was significantly higher than all other groups (controls= 0.091 ± 0.012 mA; PB= 0.068 ± 0.010 mA; sarin= 0.086 ± 0.012 mA).

Jump response: ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response was significantly lower in the sarin group (0.17 ± 0.017 mA) than in the PB (0.23 ± 0.017 mA) and PB + sarin (0.211 ± 0.016 mA) groups, but not significantly different from controls (0.19 ± 0.016 mA). At 16 weeks after treatment, ANOVA was also significant

and multiple comparisons showed that the PB+sarin group had a significantly higher threshold (0.255 ± 0.016 mA) than all other groups (controls= 0.18 ± 0.017 mA; PB= 0.152 ± 0.016 mA; sarin= 0.17 ± 0.018 mA)

Open field locomotor activity:

Parameter A (initial velocity): No statistically significant difference among treatments was found at 2 or 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for PB + sarin (360.6 ± 19.9 cm min^{-1}) was significantly higher than for the PB (272.8 ± 19.9 cm min^{-1}) group and sarin (275.3 ± 20.8 cm min^{-1}) group but not different from controls (309.5 ± 20.8 cm min^{-1}) (Fig 4).

Parameter B (habituation): No statistically significant difference among treatments was found at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter means for sarin (0.035 ± 0.0088 min^{-1}) group and PB (0.046 ± 0.0084 min^{-1}) group were lower than for controls (0.072± 0.0093 min^{-1}) while PB + sarin (0.101 ± 0.0084 min^{-1}) was significantly higher than all other groups (Fig. 4).

Total distance moved: ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (3451 ± 207 cm) was significantly lower than controls (4328 ± 338 cm). No difference vs. controls was found for the other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment.

Distance to arena's border: ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (7.78 ± 0.39 cm) was significantly lower than PB (9.58 ± 0.45 cm), and PB + sarin (9.05 ± 0.45 cm), but not different from controls (8.63 ± 0.64 cm).

Reactivity (acoustic startle): A significant increase in the average motor response in sarin-treated animals (15.3 ± 1.14 F.U.) against the controls (10.9 ± 1.14 F.U.) over the 20 trials was observed in measurements performed 2 weeks after treatment. This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (sarin = 62.6 ± 5.49 F.U.; controls 30.0 ± 5.49 F.U.; PB = 37.7 ± 5.02 F.U.; PB + sarin = 31.1 ± 5.01 F.U). In this case, the mean of the sarin group was significantly higher than all others. No difference among group means was present at 4 or 16 weeks after treatment (Fig. 5).

Passive avoidance: No difference between experimental groups was found in the time to enter the dark compartment 24 hrs after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response (data not shown).

Brain regional ChE activity: Areas rich in cholinergic nerve cells and terminals were found to have, as expected, the highest ChE activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (Table 1). Central ChE activity was not significantly modified with respect to controls at the time of measurements of tested variables. Sarin-

treated animals studied at the end of outcome variables evaluation had evidently recovered from central ChE inhibition. This is in agreement with the substantial recovery of blood ChE activity recorded for this group at about the same time after treatment (Fig.2).

Brain regional ChAT activity: Areas rich in cholinergic nerve cells and terminals were found to have, as in the case of ChE, the highest ChAT activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (data not shown).

Brain regional QNB binding: Two weeks after treatment, there was a generalized decrease in QNB binding of the sarin group, when compared with controls, that was statistically significant in caudate-putamen, hippocampus and mesencephalon (Table 2). This phenomenon reversed at 4 weeks after treatment, when a statistically significant increase in QNB binding was found in somatosensory cortex of sarin-treated animals. No statistically significant changes from control were found at 16 weeks post-treatment in any treatment group.

DISCUSSION.

Previous experimentation has shown that some functions can be affected at levels of nerve agents (such as soman and sarin) below the threshold for clinical toxicity (Chippendale et al., 1972; Russell, 1982; Wolthuis and Vanwersch, 1984). Repeated low-level exposures to soman (0.3 LD50) in rats induces initial decreases in body temperature, temporal perception, and locomotor activity. Tolerance was observed to all these effects, except soman-induced hypoalgesia. No effect of soman on memory was found by these authors (Russell et al., 1986). In another study, animals treated with low-level soman (0.4 LD50), and followed up to 6 weeks while in the treatment regime, exhibited a hyper-reactivity condition (Shih et al., 1990). In none of these cases were animals studied beyond the period of drug administration. Effects of low-dose soman on an equilibrium test in rhesus monkeys were reported to wear off 24 hrs after exposure (Switzer et al., 1990). Exposure to low-dose sarin has been recently reported to induce a decrease in activity and mobility, alteration of gait, and increase in stereotyped behavior and excitability in rats that persisted 3 to 12 months (Kassa et al., 2001a), as well as a deficit in Y-maze performance that subsided three weeks after exposure (Kassa et al., 2001b).

In the present series, the initial experiments were successful in finding reproducible effects on plasma BuChE activity of a PB concentration of 80 mg/L in the drinking water, with an estimated dose of about 10 mg/kg body mass/day. This is close to the rat equivalent (9 mg/kg body mass/day) of the dose used in humans for prophylaxis of

OP poisoning (1.2 mg/kg body mass/day), based on surface area dosage conversion (Freireich et al., 1966). The degree of plasma BuChE inhibition obtained with this dose was within the range reported for humans taking 90 mg of PB orally per 24 hrs, divided in three doses (Keeler et al., 1991).

Sarin, and PB + sarin produced more pronounced and stable inhibition of RBC AChE than did PB. AChE inhibition recovered completely by the end of the second week after discontinuation of treatment for all groups. Animals did not show signs of acute toxicity during or following treatment. The conditions established for this experimental model, i.e., exposure to the highest dose of sarin, alone or in combination with PB, devoid of acute toxicity, were thus met.

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test two weeks after the discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Russell et al., 1986; Overstreet, 1977). However, in the present experiments both blood and tissue ChE had recovered to normal levels at the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease, when compared to controls, particularly pronounced in caudate-putamen, hippocampus and mesencephalon. Down regulation of muscarinic receptors may have played a role in the behavioral phenomena described above since this was their only neurochemical correlate.

No effect of PB on locomotor activity was found. An earlier report (Hoy et al., 1999) had indicated a decrease in locomotor activity in rats given PB, but this effect was observed immediately after treatment with doses higher than used in the present study.

Both the depressed locomotor activity and enhanced reactivity induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from OP cholinesterase inhibitors lethality (Harris and Stitcher, 1984).

Previous experimentation (Servatius et al., 1998) has reported a delayed enhancement of the acoustic startle response in Wistar-Kyoto, but not Sprague-Dawley rats, with lower doses and shorter exposure times of PB than those reported here. The Wistar-Kyoto rats in those experiments were reported to have a basal plasma BuChE activity 27% lower than the Sprague-Dawley rats. These authors speculated that this fact might have caused a greater penetration of PB into the central nervous system, on account of the diminished scavenging effect of BuChE, and by that mechanism mediated the exaggerated acoustic startle response. In our experiments, we have used a dose almost ten times higher than the lower dose at which Servatius et al. reported enhancement of acoustic startle, for a longer period of time (21 days as opposed to 7), but we still did not observe any effects of PB on this response. In fact, as stated above, PB protected sarin treated animals from the delayed behavioral effects (decreased locomotor activity and hyper-reactivity) of sarin administration.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell et al., 1990), and the reverse is true of hypercholinergic states (Russell et al., 1986; Shih and Romano, 1988). A delayed elevation of nociceptive threshold for both the flinch and the jump response was found in the animals that had received PB + sarin, a phenomenon most clearly demonstrated 16 weeks after treatment. These results are difficult to interpret in the light of current knowledge of ChE inhibitors effects on pain, since no central ChE inhibition was detected at this late time. These intriguing findings deserve further exploration with other methodologies for pain threshold evaluation.

The lack of changes in the passive avoidance paradigm indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. Possible cognitive effects of the three treatments will be tested at later stages of this project by two other learning paradigms, conditioned avoidance response and Morris water maze. Learning impairments have been previously described in rats receiving PB (Liu, 1992; Shih et al., 1991). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (10 mg/kg/day), equivalent, on the basis of body surface area conversion between species, to that taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day). Moreover, in the two earlier studies referenced above, behavioral tests were performed within minutes of dosing, with no long-term follow up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of acute symptomatology, but assessment was limited to the

period immediately following treatment (Wolthuis and Vanwersch, 1984; Russell et al., 1986).

In conclusion, this study was designed to mimic the conditions of soldiers in the battlefield that are taking PB as a prophylactic treatment against nerve agents intoxication, with or without exposure to sub-symptomatic levels of these agents. PB was administered in the drinking water so as to achieve a stable dosing regime at levels adjusted to reproduce the doses used in humans. The results have shown that under these conditions, PB did not produce adverse delayed neurobehavioral effects. Moreover, at 2 weeks post-treatment, simultaneous administration of PB and sarin prevented the development of decreased exploratory activity and enhanced response to an acoustic startle test that were associated with sarin exposure without PB protection. Thus, this study gives further support to the use of PB as one of the therapeutic resources against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level nerve agent exposure. Further experimentation is planned to determine the possible effects of this treatment protocol on other physiological and neurobehavioral parameters.

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FOOTNOTES

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals,* National Research Council, 1996. The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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LEGENDS FOR FIGURES

FIGURE 1: Body mass was recorded daily (except on weekends) during the three weeks of drug treatment and the following 3 weeks. Data are averages of all animals in each experimental group: 144 rats for the first 4 weeks and 96 rats for the last 2 weeks. No statistically significant difference between groups was found.

FIGURE 2: RBC AChE during the 3 drug treatment weeks (T1-3), the pre-treatment week (two measurements, Pre 1-2) and 3 post-treatment weeks (Post 1-3). Data (Means and SE) are expressed as μmoles/ml/min. ANOVA was significant for the 3 weeks of treatment and the first week post-treatment, and multiple comparisons (Fisher LSD test, $P<0.05$) indicated that all groups were different from controls (indicated as * in figure) in all those four conditions.

FIGURE 3, Top panel: Means and SE of flinch nociceptive threshold for all experimental groups used (12 rats per group). ANOVA was significant at 16 weeks after treatment and multiple comparisons (Fisher LSD test, $P<0.05$) indicated that the sarin+PB mean was significantly higher than controls (indicated as * in figure) and all other treatments at that time. **Bottom panel**: Means and SE of jump nociceptive threshold for all experimental groups used (12 rats per group). ANOVA was significant at 4 weeks and 16 weeks . At 4 weeks, multiple comparisons indicated that the sarin mean was lower than the PB and sarin+PB means, but not different from controls. At 16 weeks after treatment, the sarin+PB mean was significantly higher than controls (indicated as * in figure) and all other groups.

FIGURE 4: Means and SE of parameters A (initial velocity) and B (habituation) in non-linear fits of open field exploratory activity for all groups (12 rats per group). **Top panel**: ANOVA was significant for initial velocity and multiple contrasts (Fisher LSD test, $P<0.05$) indicated that sarin + PB was significantly higher than the PB and the sarin groups but not different from controls. **Bottom panel**: ANOVA was significant only at week 16 for habituation, and multiple contrasts indicated that sarin and PB alone were lower than controls (indicated as * in figure), while sarin+PB was significantly higher than controls and all other groups.

FIGURE 5, Top panel: Means and SE of average acoustic startle response across trials for all experimental groups used (12 rats per group). ANOVA was significant at 2 weeks only. Multiple comparisons (Fisher LSD test, $P<0.05$) indicated that the sarin mean was higher than controls (indicated as * in figure). **Bottom panel**: ANOVA was also significant only at 2 weeks for the maximal response over 20 consecutive trials. The sarin mean was higher than controls and all other groups.

Table 1: Acetylcholinesterase activity (nanomoles/mg tissue/min). Data shown are mean ± S.E. of 12 animals per experimental condition and time post-treatment

2 weeks post-treatment

	Control	PB	Sarin	Sarin+PB
Somat sens Ctx	7.8 ± 0.5	7.4 ± 0.3	7.2 ± 0.7	6.3 ± 0.2
Temporal Ctx	7.7 ± 0.3	7.0 ± 0.2	7.0 ± 0.3	5.9 ± 0.5
Piriform Ctx	17.9 ± 1.0	18.8 ± 1.4	18.4 ± 1.5	17.7 ± 1.3
Hippocampus	11.5 ± 0.5	11.6 ± 0.2	10.3 ± 0.7	10.7 ± 0.6
Caudate-Putamen	73.5 ± 3.1	68.7 ± 3.4	67.0 ± 4.4	67.1 ± 4.3
Thalamus	15.7 ± 0.6	12.5 ± 0.7	11.3 ± 0.7	12.7 ± 1.0
Hypothalamus	13.0 ± 1.2	12.3 ± 1.0	12.9 ± 0.8	10.8 ± 0.7
Mesencephalon	16.2 ± 1.1	16.5 ± 0.6	15.0 ± 1.1	15.7 ± 0.5
Cerebellum	4.4 ± 0.2	4.1 ± 0.3	4.4 ± 0.3	3.8 ± 0.3
Medulla	13.4 ± 0.7	13.4 ± 0.7	13.2 ± 0.8	12.3 ± 1.2

4 weeks post-treatment

	Control	PB	Sarin	Sarin+PB
Somat sens Ctx	7.3 ± 0.3	7.0 ± 0.1	7.4 ± 0.2	7.1 ± 0.3
Temporal Ctx	7.0 ± 0.4	7.2 ± 0.1	8.1 ± 0.3	7.0 ± 0.4
Piriform Ctx	18.6 ± 1.2	18.2 ± 1.3	18.5 ± 0.8	19.3 ± 1.2
Hippocampus	10.6 ± 0.8	12.0 ± 0.2	12.4 ± 0.6	12.4 ± 0.4
Caudate-Putamen	66.0 ± 4.4	74.1 ± 3.4	66.7 ± 2.9	67.2 ± 6.3
Thalamus	18.3 ± 3.8	19.2 ± 5.4	15.9 ± 0.7	14.8 ± 0.7
Hypothalamus	10.9 ± 0.9	11.8 ± 0.3	12.9 ± 0.5	11.2 ± 0.4
Mesencephalon	16.5 ± 0.5	17.2 ± 0.6	17.9 ± 0.8	17.2 ± 0.4
Cerebellum	4.6 ± 0.2	4.8 ± 0.1	4.3 ± 0.4	6.2 ± 1.2
Medulla	12.9 ± 1.2	14.2 ± 0.4	15.7 ± 0.4	15.9 ± 1.0

16 weeks post-treatment

	Control	PB	Sarin	Sarin+PB
Somat sens Ctx	7.6 ± 0.9	7.4 ± 0.2	7.3 ± 0.5	7.2 ± 0.2
Temporal Ctx	7.6 ± 0.4	7.3 ± 0.1	6.6 ± 0.2	6.8 ± 0.2
Piriform Ctx	15.8 ± 1.9	18.3 ± 0.7	17.3 ± 0.8	20.9 ± 1.7
Hippocampus	10.0 ± 0.8	10.4 ± 0.3	10.0 ± 0.5	10.8 ± 0.7
Caudate-Putamen	63.4 ± 5.4	71.4 ± 1.7	61.3 ± 2.6	70.2 ± 2.3
Thalamus	12.7 ± 1.0	13.9 ± 0.5	12.7 ± 0.4	11.9 ± 0.9
Hypothalamus	10.7 ± 0.4	11.0 ± 0.3	11.1 ± 0.6	10.2 ± 0.6
Mesencephalon	15.9 ± 0.9	16.5 ± 0.3	14.4 ± 1.1	16.3 ± 0.4
Cerebellum	5.1 ± 0.5	4.5 ± 0.1	4.3 ± 0.2	4.7 ± 0.1
Medulla	13.2 ± 0.5	13.7 ± 0.4	12.8 ± 0.7	13.4 ± 0.4

Table 2: ^{3}H-QNB binding (fmoles/mg tissue). Data shown are mean ± S.E. of 12 animals per experimental condition and time post-treatment.

2 weeks post-treatment

	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	132.4	± 9.5	125.4	± 6.1	112.2	± 11.2	114.6	± 10.8
Temporal Ctx	125.4	± 5.0	125.6	± 4.2	96.7	± 14.5	105.6	± 7.5
Piriform Ctx	121.8	± 7.9	107.5	± 2.8	93.2	± 12.9	98.0	± 5.9
Hippocampus	115.9	± 3.5	114.4	± 4.7	92.2	± 10.5*	95.7	± 6.6
Caudate-Putamen	177.8	± 12.9	171.1	± 7.6	128.8	± 15.7*	158.2	± 12.1
Thalamus	68.6	± 3.5	61.6	± 1.5	60.1	± 8.4	53.9	± 4.2
Hypothalamus	42.1	± 5.0	38.3	± 1.4	29.3	± 4.4	36.5	± 3.0
Mesencephalon	48.9	± 2.3	42.9	± 1.7	32.5	± 4.4*	44.3	± 3.6
Cerebellum	9.9	± 0.7	10.4	± 1.0	6.2	± 1.2	9.4	± 1.3
Medulla	36.4	± 1.6	35.7	± 1.4	36.7	± 8.1	35.8	± 3.0

4 weeks post-treatment

	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	125.9	± 7.2	123.9	± 3.9	131.9	± 5.2*	107.8	± 6.9
Temporal Ctx	121.1	± 6.4	123.7	± 2.7	121.3	± 10.3	106.1	± 6.0
Piriform Ctx	111.7	± 4.6	111.7	± 3.2	110.9	± 6.3	107.2	± 4.2
Hippocampus	105.4	± 5.7	118.0	± 3.2	107.9	± 8.3	105.8	± 6.3
Caudate-Putamen	170.4	± 6.5	182.0	± 4.5	187.5	± 9.7	160.2	± 6.2
Thalamus	64.1	± 3.1	61.4	± 1.8	58.8	± 3.0	60.1	± 3.3
Hypothalamus	33.3	± 2.4	39.4	± 1.8	39.5	± 1.8	36.0	± 3.5
Mesencephalon	51.3	± 3.0	44.3	± 1.5	48.1	± 1.7	45.4	± 3.2
Cerebellum	10.1	± 0.8	9.5	± 0.4	10.1	± 0.8	10.2	± 1.3
Medulla	36.9	± 3.5	35.1	± 2.3	41.2	± 2.5	43.0	± 3.4

16 weeks post-treatment

	Control		PB		Sarin		Sarin+PB	
Somat sens Ctx	97.1	±9.2	108.3	±4.0	116.3	±11.6	101.4	±4.2
Temporal Ctx	113.3	±4.7	117.4	±3.5	118.8	±8.6	117.1	±5.0
Piriform Ctx	102.2	±2.6	108.1	±3.9	109.5	±7.9	95.6	±3.4
Hippocampus	109.2	±5.7	120.4	±5.2	109.9	±8.5	115.3	±2.4
Caudate-Putamen	159.8	±7.5	167.8	±3.2	157.1	±11.3	163.4	±7.7
Thalamus	56.8	±2.9	61.8	±2.1	56.6	±3.8	59.9	±3.4
Hypothalamus	37.1	±1.7	35.9	±1.8	38.7	±3.2	29.2	±1.1
Mesencephalon	36.5	±4.0	41.9	±0.8	45.4	±3.6	36.1	±1.2
Cerebellum	10.8	±2.2	7.9	±0.5	10.7	±1.0	9.5	±0.4
Medulla	30.4	±1.2	32.3	±1.2	37.1	±2.8	30.2	±1.6

* Statistically significant by ANOVA and Fisher's LSD tests.

ACKNOWLEDGEMENTS.

We thank Jaclyn D'Elia and Leah Rechen for invaluable technical help.

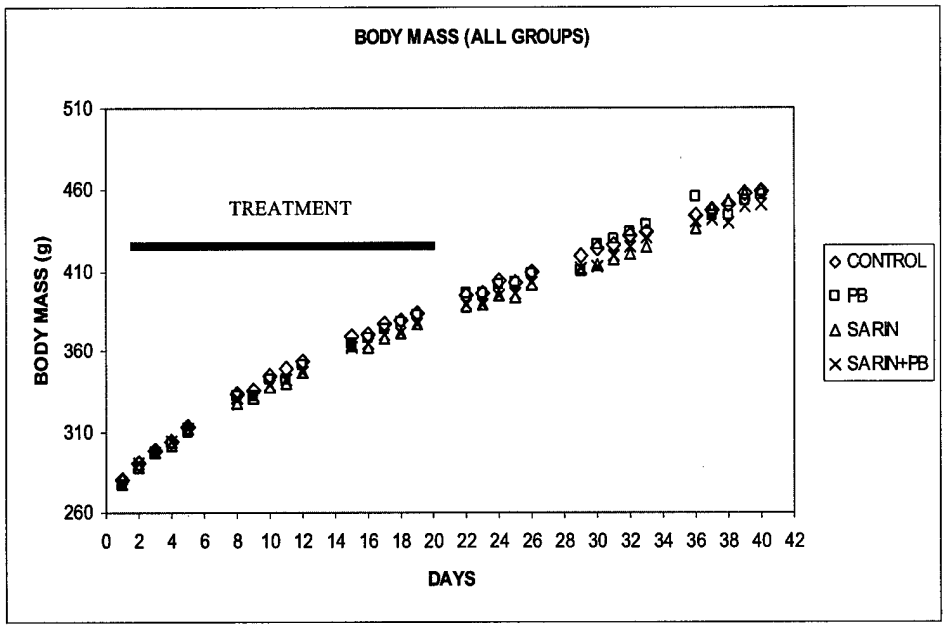

BODY MASS (ALL GROUPS)
510
460
410
360
310
260
BODY MASS (g)
TREATMENT
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
DAYS
CONTROL
PB
SARIN
SARIN+PB

FIGURE 1

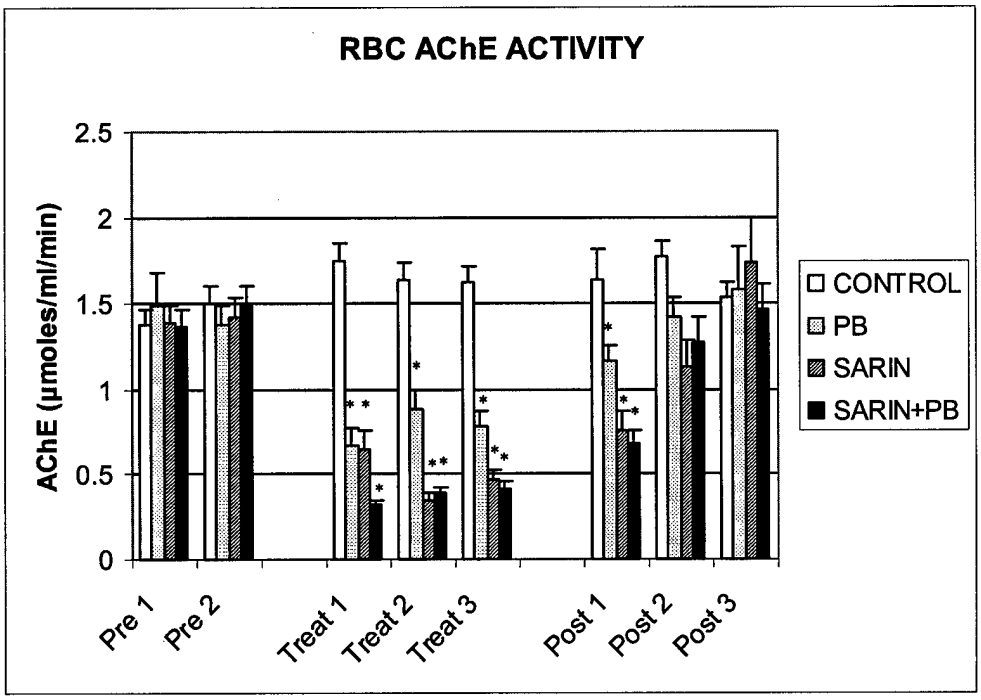

RBC AChE ACTIVITY
AChE (µmoles/ml/min)
2.5
2
1.5
1
0.5
0
Pre 1
Pre 2
Treat 1
Treat 2
Treat 3
Post 1
Post 2
Post 3
CONTROL
PB
SARIN
SARIN+PB

FIGURE 2

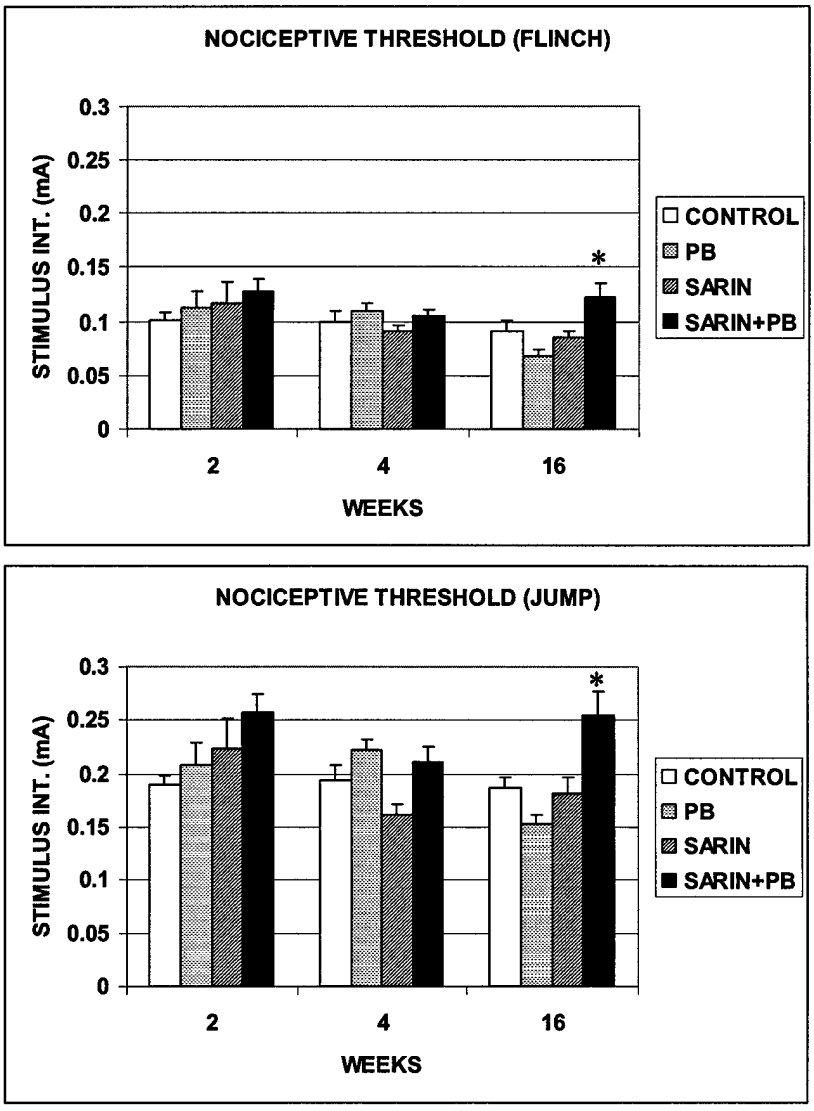
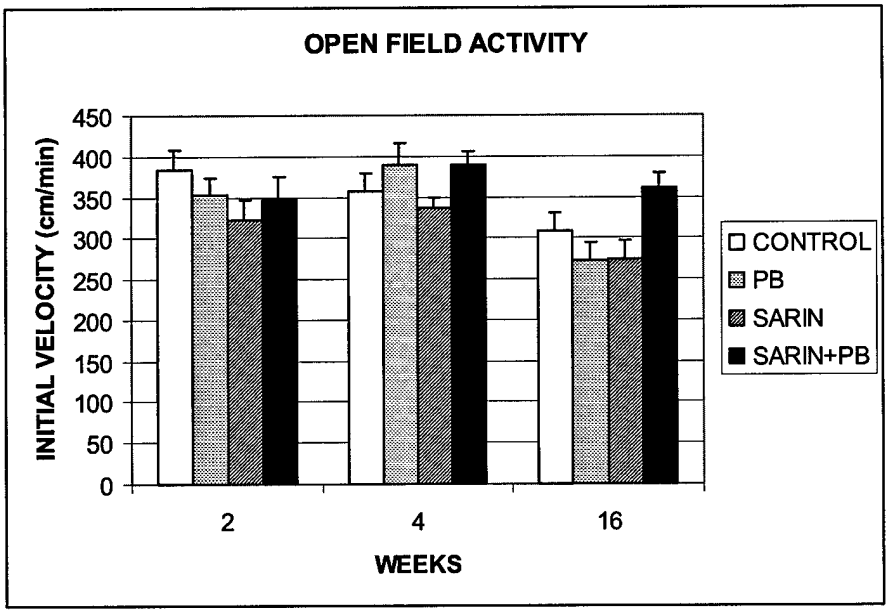

NOCICEPTIVE THRESHOLD (FLINCH)
STIMULUS INT. (mA)
0.3
0.25
0.2
0.15
0.1
0.05
0
2
4
16
WEEKS
*
CONTROL
PB
SARIN
SARIN+PB
NOCICEPTIVE THRESHOLD (JUMP)
STIMULUS INT. (mA)
0.3
0.25
0.2
0.15
0.1
0.05
0
2
4
16
WEEKS
*
CONTROL
PB
SARIN
SARIN+PB

FIGURE 3


OPEN FIELD ACTIVITY
INITIAL VELOCITY (cm/min)
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350
300
250
200
150
100
50
0
2
4
16
WEEKS
CONTROL
PB
SARIN
SARIN+PB

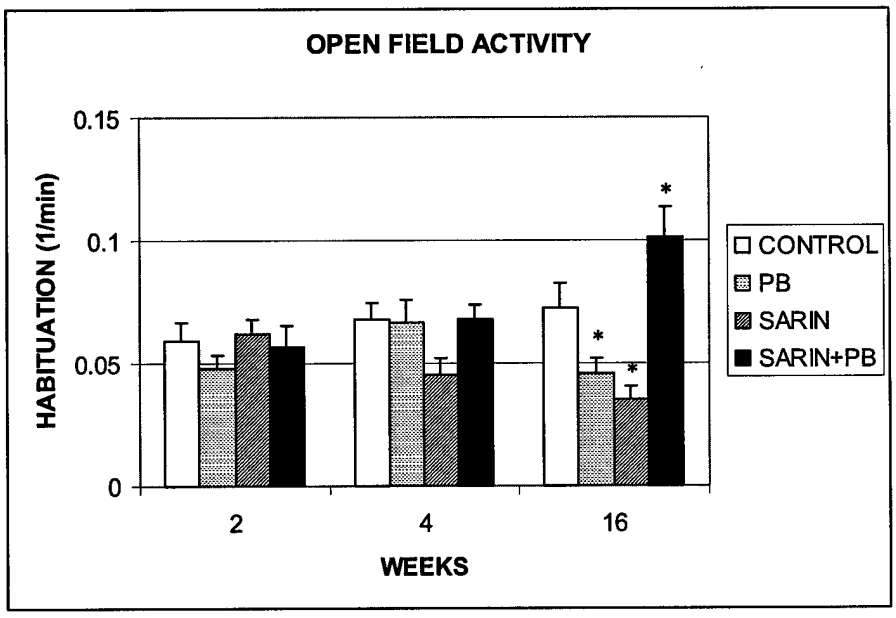
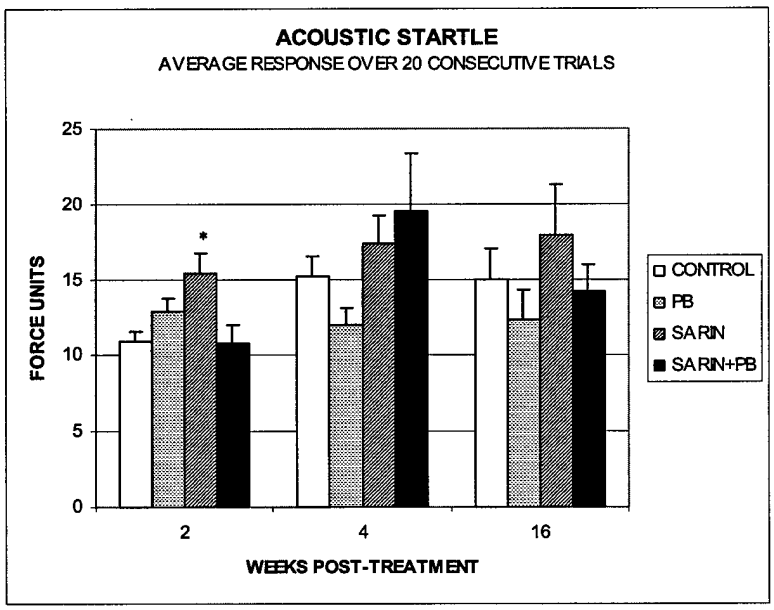

OPEN FIELD ACTIVITY
HABITUATION (1/min)
0.15
0.1
0.05
0
*
*
*
2
4
16
WEEKS
CONTROL
PB
SARIN
SARIN+PB

FIGURE 4


ACOUSTIC STARTLE
AVERAGE RESPONSE OVER 20 CONSECUTIVE TRIALS
FORCE UNITS
25
20
15
10
5
0
*
2
4
16
WEEKS POST-TREATMENT
CONTROL
PB
SARIN
SARIN+PB

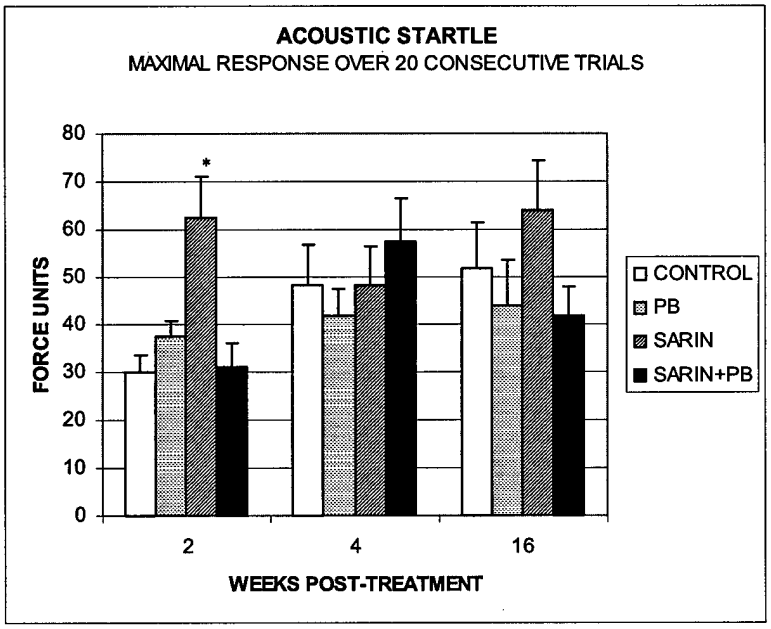

ACOUSTIC STARTLE
MAXIMAL RESPONSE OVER 20 CONSECUTIVE TRIALS
FORCE UNITS
80
70
60
50
40
30
20
10
0
*
2
4
16
WEEKS POST-TREATMENT
CONTROL
PB
SARIN
SARIN+PB

FIGURE 5

EFFECTS OF LOW-DOSE CHOLINESTERASE INHIBITORS ON COGNITION.

Oscar U. Scremin [1,3], Tsung-Ming Shih [2], Ly Huynh [1], Margareth Roch [1], Ruth Booth [1], Jaclyn D'Elia [2], Christine Cable [2], Anjana Sreedharan [1] and Donald J. Jenden [4]

1. Research, VA GLA Healthcare System, Los Angeles CA, USA; 2. Neurotoxicology Branch, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, USA; 3.Physiology, 4. Medical and Molecular Pharmacology, UCLA School of Medicine, Los Angeles, CA, USA.

Abstract

Veterans from the Persian Gulf War complain of neurological and cognitive dysfunction, ascribed by some authors to pyridostigmine bromide (PB) and/or sarin exposure. In the present experiments, passive (PA) and conditioned (CA) avoidance learning and habituation (HAB) of exploration of a novel environment were used to assess cognition in male Sprague-Dawley (Crl:CD(SD)IGSBR) rats at 2, 4, and 16 weeks after exposure to non-toxic doses of PB and sarin alone or in combination. Measured parameters were retention time 48 hrs after conditioning (PA), criterion (6 consecutive avoidances), escape and avoidance time on two tests on consecutive days (CA), and the decay slope of exploratory activity (HAB). The results have shown that under these conditions, PB did not produce adverse delayed cognitive effects, other than a delayed decrease in habituation of exploratory activity in the open field. A similar effect was observed with sarin and the opposite effect for the combination of sarin with PB. No effect of any of the treatments could be found in the conditioned or passive avoidance tests. Thus, this study does not support the hypothesis that delayed cognitive impairments experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level nerve agent exposure.

In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

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Introduction.

Carbamate cholinesterase inhibitors provide additional protection, when used as pretreatment, from exposure to soman and tabun than that afforded by atropine and oxime alone (Dirnhuber et al., 1979) (Leadbeater et al., 1985) (Koplovitz et al., 1992) (Kluwe et al., 1987). On the basis of these findings, the quaternary cholinesterase inhibitor pyridostigmine bromide (PB) was adopted by USA and NATO armies as wartime pretreatment adjunct for nerve agent exposure. The therapeutic target for this application of pyridostigmine has been to maintain inhibition of plasma butyryl-cholinesterase (BuChE) between 20% to 40%. Large scale use of this premedication occurred during the Persian Gulf War, with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler et al., 1991). This pretreatment and the possible exposure to low level sarin have been proposed by some to contribute to a conglomerate of symptoms experienced by Persian Gulf War veterans. The present study was designed to determine whether sub-symptomatic exposure to PB or low-dose sarin, alone or in combination, could elicit cognitive changes detectable 2 to 16 weeks after exposure to the agent.

Methods

Adult male Sprague-Dawley rats were used. Preliminary experiments were conducted to determine the optimal dose of sarin (the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment) and PB (the dose producing 20-30% inhibition of plasma BuChE, the degree of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War). Experiments were conducted at the US Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC-I.

Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman using the appropriate substrates.

Animals were treated for three weeks with (1) subcutaneous (s.c.) saline injection, (2) PB in drinking water (80 mg/L), (3) sarin 0.5 x LD50 three times/week s.c. injection, or (4) PB in drinking water plus sarin s.c.. There were 36 animals in each group, with three subgroups of 12 in each treatment that were studied 2, 4 or 16 weeks after treatment.

After tests for passive and active avoidance conditioning and open field activity were completed, rats were euthanized and the brain regions of interest were microdissected from frozen brain slices. These regions were homogenized, and aliquots were used for determination of tissue AChE activity (Ellman et al., 1961), ChAT activity (Fonnum, 1975), and quinuclydinyl benzilate (QNB) binding with saturation assays (Yamamura and Snyder, 1974).

Inhibited (passive) avoidance response: This response was measured in a "step through" apparatus (McGaugh, 1972), consisting of (a) a small compartment made of white plastic, (b) a larger, dark compartment of stainless steel, and (c) a shock delivery unit adjustable for the intensity and duration of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 hrs. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment leads immediately to the closing of a door and administration of footshock. Retention was tested after a 48-hr delay, the measure being time taken to enter the dark compartment after release from the white compartment. The time to enter was defined as "retention," a measure of memory of the single training session. The retention trials were set at a limit of 10 min. The times for animals not entering during the 10 min were recorded as 600 sec.

Conditioned avoidance response: A discrete trial, one-way conditioned avoidance response was observed using the apparatus and general procedure described by Russell and Macri (Russell and Macri, 1979). Two responses were studied: an innate escape response and a learned avoidance response. There was a maximum of 30 trials per session, with two sessions 24 hrs apart. The number of animals reaching criterion (6 consecutive avoidance responses) and the average escape and avoidance times per animal in both sessions were recorded for all experimental groups.

Open field locomotor activity: Activity was measured during a 20-min session in circular open field chambers of 60 cm diameter under low level red light illumination. This was done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. Each animal's movements were recorded with a video tracking and motion analysis system, consisting of a Sony CCD video camera (sensitive to the wavelenth of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory (Figs 1,2). Distance traveled was summated at 1-min intervals, and these values were fitted by non-linear regression, using the Marquardt algorithm, to the model:

$$Y = A \cdot e^{-Bt} \quad (1)$$

where Y = distance moved (cm) and t= time after initiation of test (min). The values of parameters A (initial velocity, cm min^{-1}) and B (habituation, min^{-1}) were obtained as described above for every animal (Fig.3). Analysis of variance (ANOVA) was then performed for the two parameters using factors treatment (control, PB, sarin and sarin+PB) and time after treatment (2, 4, and 16 weeks). In addition, total distance traveled and mean distance to the arena's border (the wall of the chamber) during the entire test were also calculated for every animal.

Data Analysis: Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with standard errors (SE). Differences between group means were tested by ANOVA (general linear model) followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method.

Results

Dose Determination Studies: The LD50 of sarin was determined to be 125 µg/kg, sc. An initial evaluation indicated that animals whose drinking water contained PB at a concentration of 80 mg/L had inhibition of plasma BuChE slightly greater than 20% on average, and was within the target effect set for these experiments (20 to 30% inhibition). The next higher PB concentration in drinking water (160 mg/L) induced a larger plasma BuChE inhibition (between 27 and 40 %). Thus the concentration of 80 mg/L PB in drinking water was adopted for the rest of the study. No sign of toxicity, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination), was found in animals drinking water containing PB during the three-week treatment periods. The dose finding for sarin and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose devoid of acute toxic effects, as described above, when given alone or in combination with PB (80 mg/L) in drinking water.

Body mass: Means of body mass, recorded daily on weekdays during the three weeks of treatment and the post-treatment weeks showed the expected increase with age, but no statistically significant differences were found among treatments.

Blood ChE activity: Measurements of red blood cells (RBC) AChE during the 3 drug treatment weeks, the pre-treatment week (two measurements) and 3 post-treatment weeks are shown in Fig 1. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the three weeks of treatment. Sarin and sarin plus pyridostigmine produced an average decrease in RBC AChE to 35% and 27% of pre-treatment, respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

Open field locomotor activity:

Parameter A (initial velocity): No statistically significant difference was found among treatments at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for sarin plus PB (360.6 ± 19.9 cm min^{-1}) was significantly higher than the PB (272.8 ± 19.9 cm min^{-1}) and sarin (275.3 ± 20.8 cm min^{-1}) groups but not different from controls (309.5 ± 20.8 cm min^{-1}) (Fig 5).

Parameter B (habituation): No statistically significant difference was found among treatments at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter

means for sarin (0.035 ± 0.0088 min^{-1}) and PB (0.046 ± 0.0084 min^{-1}) were lower than for controls (0.072 ± 0.0093 min^{-1}), while sarin+PB (0.101 ± 0.0084 min^{-1}) was significantly higher than for all other groups (Fig. 4).

Passive avoidance: No difference between experimental groups was found in the time to enter the dark compartment 24 hrs after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response.

Conditioned avoidance: Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test and the same parameters for animals that gained or lost criterion in the second day with regard to the first are shown in Figs 5 and 6. No significant difference was detected among experimental groups for the pooled data shown in the Fig.5, nor for any of the time points after treatment.

Brain regional AChE and ChAT activities were not affected at any time after treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon, 2 weeks after exposure to sarin.

Discussion

This study was designed to mimic the conditions of soldiers in the battlefield taking PB as a prophylactic treatment against nerve agent intoxication, with or without exposure to subsymptomatic levels of these agents. PB was administered in the drinking water to achieve a stable dosing regime at levels adjusted to reproduce the doses used in humans.

The lack of changes in the passive and conditioned avoidance paradigms under the conditions of this experimental model indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. On the other hand, habituation in the open field test, considered a primitive form of learning, was impaired for the PB and sarin groups at 16 weeks after treatment. This phenomenon was enhanced, however, in the group in which sarin treatment was combined with PB at the same time point. Given the present evidence, these phenomena are difficult to interpret and may require exploration of longer time points after treatment to define the possible interaction between sarin and PB on this particular type of behavior. Possible cognitive effects of the three treatments will be tested at later stages of this project by another learning test involving spatial orientation, the Morris water maze.

Learning impairments have been previously described in rats receiving PB (Liu, 1992) (Shih et al., 1991). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (11 mg/kg/day) or taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day) (Keeler, Hurst, and Dunn, 1991). Moreover, in these two earlier studies behavioral tests were performed within minutes of dosing, with no long-term follow up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of symptomatology, but assessment was limited to the period immediately following treatment (Wolthuis and Vanwersch, 1984; Russell et al., 1986).

In conclusion, the data obtained in this study on avoidance learning paradigms does not support the hypothesis that delayed cognitive impairments experienced by Persian Gulf War veterans could be due to PB, either alone or in association with low-level nerve agent exposure. The issue of possible effects of sarin or PB, and their interaction on the primitive form of learning represented by habituation on the open field test, deserves further exploration with other tests of spatial orientation.

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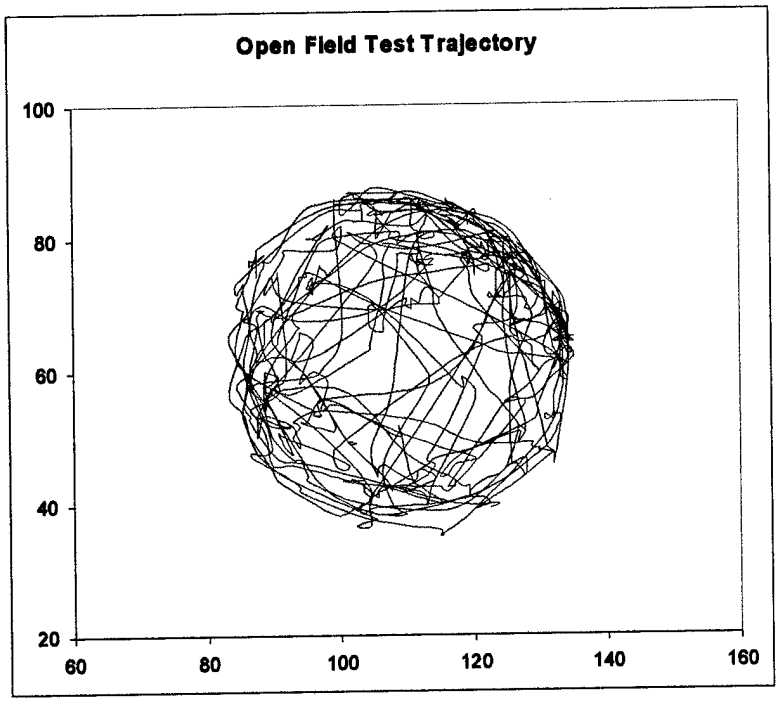

Figure 1: Open field test: trajectory of one animal over the circular arena as tracked by the video-monitoring system.

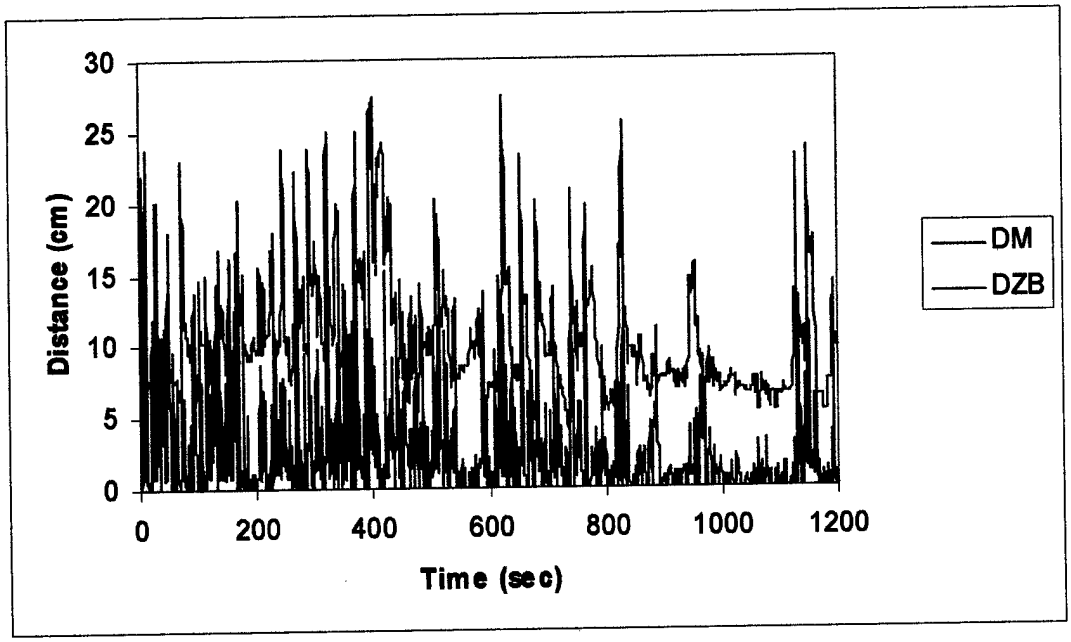

Figure 2: Open field test: distance moved (DM) and distance to arena's border (DZB) were computed every second throughout the test for the experiment shown in Fig. 1

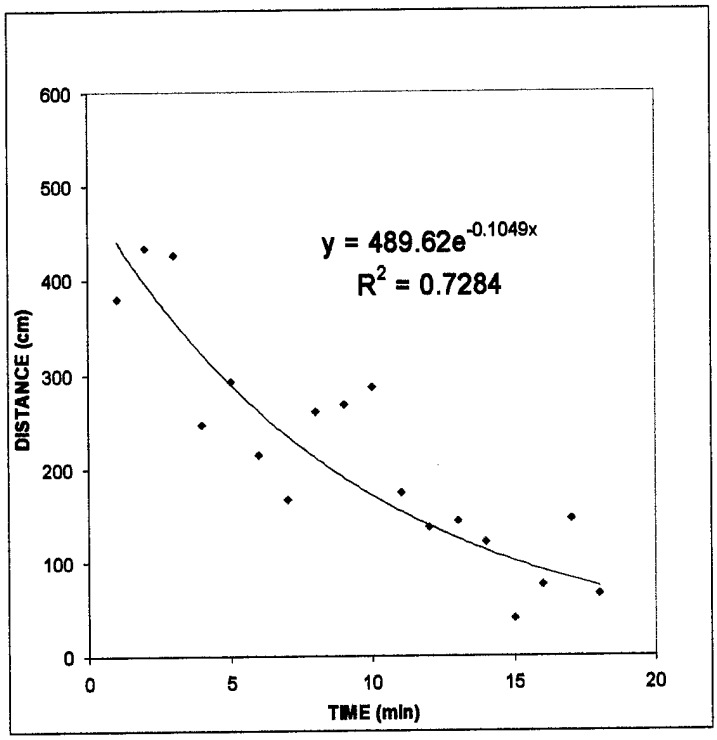

Figure 3: Parameters of monoexponential fits of distance moved over time in an open field (shown here for a single animal) were obtained in every case and calculated as described in Methods.

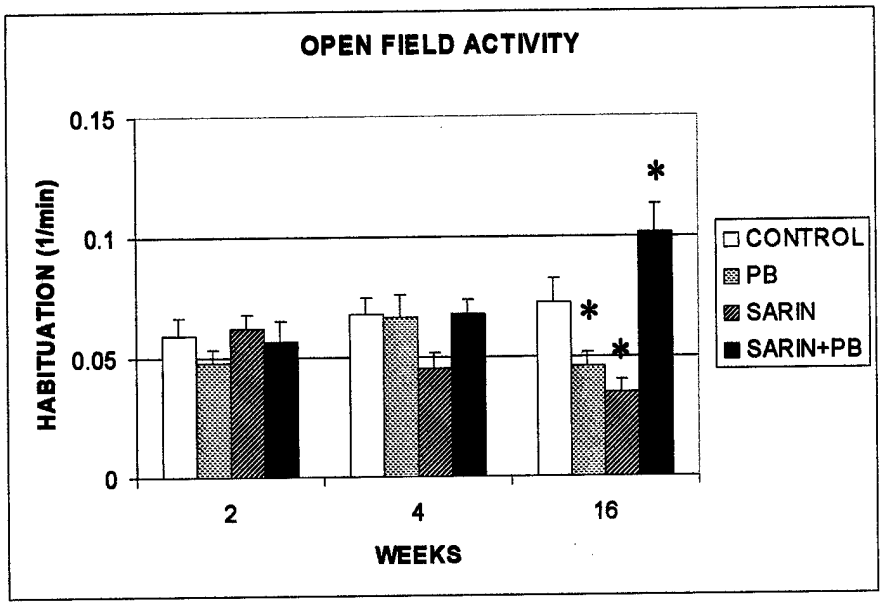

Figure 4: Means and SE of parameter B (habituation, rate constant). At 16 weeks following exposure, sarin and PB were lower than controls ($P < 0.01$ and 0.05 respectively), while sarin+PB was significantly higher than all other groups ($P < 0.001$ vs. sarin and PB, and $P < 0.025$ vs. controls). Parameter A (initial velocity, Y intercept) showed no differences among groups.

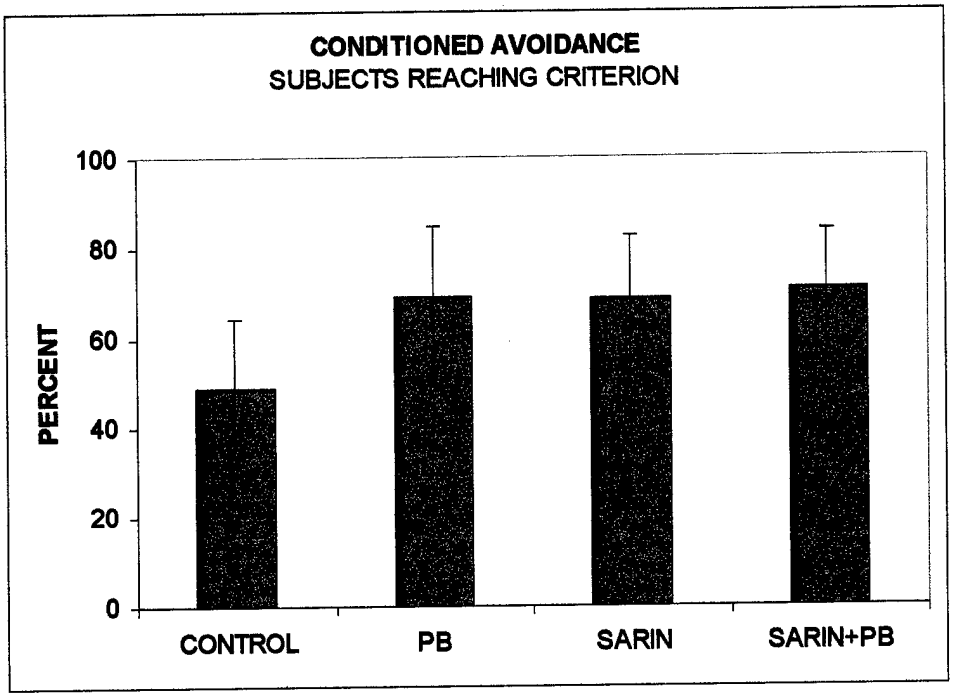

Figure 5: Percentage and 95% confidence intervals of animals reaching criterion (6 consecutive avoidances) in the 2nd day of the conditioned avoidance test. There were no statistically significant differences between groups (pooled data from all times after treatment).

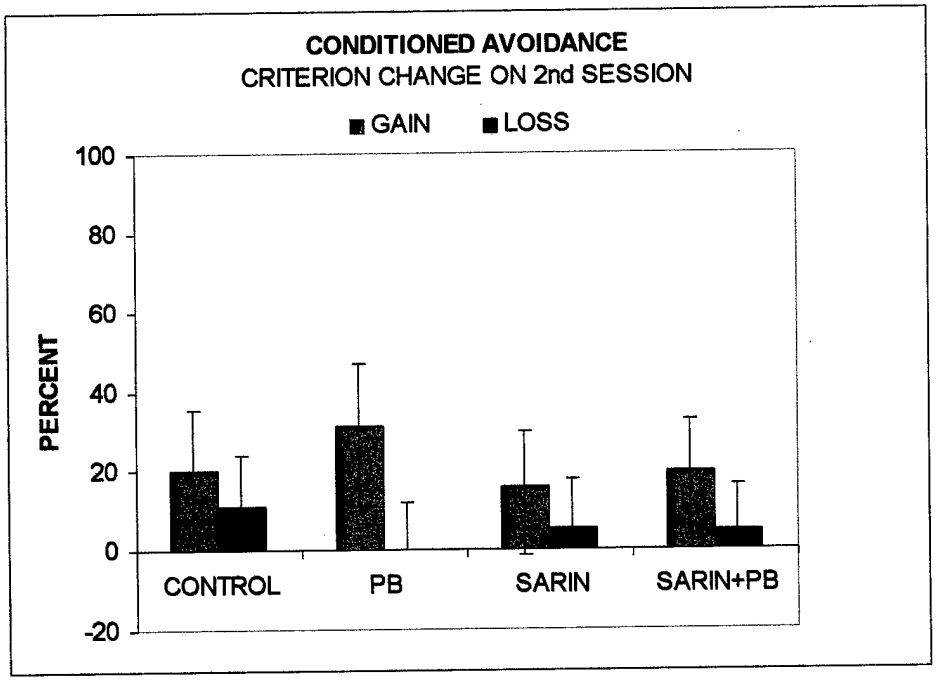

Figure 6: Percent and 95% confidence intervals of animals that gained or lost criterion in the second day when compared with the first. Pooled data from 2, 4, and 16 weeks after exposure.

PYRIDOSTIGMINE BROMIDE PREVENTS DELAYED NEUROLOGICAL EFFECTS OF LOW DOSE SARIN.

Oscar U. Scremin [1,3], Tsung-Ming Shih [2], Ly Huynh [1], Margareth Roch [1], Ruth Booth [1], Jaclyn D'Elia [2], Christine Cable [2], Anjana Sreedharan [1] and Donald J. Jenden [4]

1. Research, VA GLA Healthcare System, Los Angeles CA, USA; 2. Neurotoxicology Branch, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, USA; 3.Physiology, 4. Medical and Molecular Pharmacology, UCLA School of Medicine, Los Angeles, CA, USA.

Abstract

Many veterans of the Persian Gulf War complain of neurological symptoms, including balance disturbances, vertigo, and muscle aches and weaknesses, which have been ascribed by some authors, among other possible factors, to exposure to the ChE inhibitors pyridostigmine bromide (PB) and/or sarin. The hypothesis that these agents, alone or in combination, elicit delayed neurological dysfunction was tested in Sprague-Dawley rats (Crl:CD(SD)IGSBR). Acoustic startle, locomotor activity in an open field, nociceptive threshold, and neural cardiovascular regulation were studied 2, 4, and 16 weeks after exposure to sub-toxic doses of PB and sarin, alone or in combination. Brain regional acetylcholinesterase (AChE) and cholinacetyltransferase (ChAT) activities and muscarinic receptor binding were studied in 10 critical brain regions. Two weeks after sarin, acoustic startle was enhanced, while distance explored in the open field decreased. These effects were absent with PB plus sarin or PB by itself. No effect on any variable was found at 4 weeks, while at 16 weeks an elevation of nociceptive threshold was found with the combination of sarin+PB. Mean arterial blood pressure, heart rate and gain of the baroreceptor reflex were similar across treatments. Brain regional AChE and ChAT activities were not affected at any time after any treatment, but muscarinic receptors were down-regulated in hippocampus, caudate-putamen and mesencephalon at 2 weeks. In conclusion, PB protected against neurologic dysfunction in animals exposed to low dose sarin.

In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

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Introduction

Exposure to pyridostigmine bromide (PB) and/or sarin has been implicated by some authors in the causation of a complex conglomerate of symptoms suffered by veterans of the Persian Gulf War (Haley, 2001). Exposure to PB resulted from its use as a prophylactic of nerve agent intoxication (Dirnhuber et al., 1979;Leadbeater et al., 1985;Koplovitz et al., 1992;Kluwe et al., 1987;Keeler et al., 1991). Large scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler, Hurst, and Dunn, 1991). Possible exposure to sarin may have occurred following explosions of ammunition dumps with consequent air contamination at Khamisiyah, Iraq (McCauley et al., 2001).

This study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf war veterans, could elicit delayed and persistent neurological dysfunction in experimental animals. An open field activity test was used to assess motor activity. Auditory startle and nociceptive threshold were assessed to determine the existence of possible dysfunction of the somatic nervous system since they have been shown to be affected by acute cholinesterase inhibition (Philippens et al., 1997;Russell et al., 1986). The baroreceptor mechanism of arterial blood pressure control was tested as an indicator of autonomic nervous system function because it includes both peripheral and central cholinergic steps in its circuitry (Brezenoff and Giuliano, 1982;Higgins et al., 1973). In addition, we analyzed, in relevant brain regions, the activity of ChAT and AChE, the enzymes responsible for ACh synthesis and degradation respectively, as well as the expression of muscarinic cholinergic receptors. These assays were performed in the same animals that were subjected to the tests mentioned above.

Methods

Animals: Adult male Sprague-Dawley rats were used. Preliminary experiments were conducted to determine the optimal dose of sarin (the highest dose not associated with toxic signs following single or multiple doses within the three-week period of treatment) and PB (the dose producing 20-30% inhibition of plasma BuChE, the degree of butyrylcholinesterase (BuChE) inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War).

Experiments were conducted at the US Army Medical Research Institute of Chemical Defense (USAMRICD) or the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by AAALAC. Animals were treated during three weeks with (1) subcutaneous (s.c.) saline injection, (2) PB in drinking water (80 mg/L), (3) sarin 0.5 x LD50 three times/week s.c. injection, or (4) PB in drinking water plus sarin s.c.

Open field locomotor activity: This was measured during a 20-min session in circular open field chambers of 60 cm diameter under low level red light illumination. This was done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a CCD video camera (Sony, Inc.), sensitive to the wavelenth of light used, Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Total distance traveled and mean distance to the arena's border (the wall of the chamber) during the entire test were calculated for every animal.

Reactivity (startle response): Reactivity is defined as a response to a sudden brief and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been previously described (Silverman et al., 1988); (Russell and Macri, 1979). In this procedure the animals stand unrestrained on a platform provided with a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom designed computer program delivers a controlled sound and integrates and digitizes the movement-related electrical signal. Quantification of the response is provided in arbitrary force units. In the currently reported experiments, 20 trials were performed at fixed intervals of 10 seconds.

Nociceptive threshold: The procedure to measure nociceptive threshold used in these experiments has been previously described (Crocker and Russell, 1984) and utilizes reaction to a mild electric foot shock as its measure. It involves the "up and down" method described by Dixon (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 sec at 10-sec intervals. The shock intensities were available in a range from 0.05 mA to 4.0 mA and arranged in a $\log_{10}$ scale at 0.1 $\log_{10}$ units. Shock levels were set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animals response on each trial. A "flinch" was defined as an elevation of 1 or 2 paws from the grid floor and "jump" as rapid withdrawal of three or more paws from the grid.

Cardiovascular regulation: Animals were instrumented with arterial and venous femoral indwelling catheters under halothane anesthesia for recording of arterial blood pressure and infusion of drugs respectively. They were then allowed to recover from anesthesia in a Bollman cage, where they remained conscious but restrained during the rest of the test. Arterial blood pressure (BP) was transiently altered by pulse injection of phenylephrine (5 to 10 µg/kg, i.v.) and sodium nitroprusside (20 to 50 µg/kg, i.v.). Heart rate (HR) was continuously recorded along with arterial blood pressure, and regressions of HR on BP were calculated from data obtained before and after the pulse injections of phenylephrine and nitroprusside, as an estimate of the baroreceptor gain.

Neurochemistry: Whole blood and RBC AChE activity as well as plasma BuChE were determined by an adaptation of the method of Ellman using the appropriate substrates. After the tests described above were completed, rats were euthanized, and the following brain tissue regions were microdissected from frozen brain slices: somato-sensory, temporal, and pyriform cortex, hippocampus, caudate-putamen, thalamus, hypothalamus, mesencephalon, cerebellum, and medulla. These regions were homogenized, and aliquots used for determination of tissue AChE activity (Ellman et al., 1961), ChAT activity (Fonnum, 1975), and quinuclydinyl benzilate (QNB) binding with saturation assays (Yamamura et al., 1974).

Experimental groups: Animals were divided into 4 groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline. Group 2 animals received PB in drinking water (80 mg/L) and were injected with saline. Group 3 animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to 0.5 LD50). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above. PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 08:00 hour. At 09:00 that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 17:00 hours on Friday of the third week. There were 36 animals in each group, with three subgroups of 12 in each treatment group that were studied 2, 4 or 16 weeks after treatment.

Data Analysis: Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with standard errors (SE) except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher's least significant difference method.

Results

Immediate treatment effects.

The dose finding for sarin, and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose devoid of acute toxic effects, as described above, when given alone or in combination with PB (80 mg/L in drinking water). Means of body mass, recorded daily during weekdays, through the three weeks of treatment showed the expected increase with age, but no statistically significant differences were found among treatments.

PB induced a pronounced decrease in RBC AChE activity during the first week, which recovered partially during the following two weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the

three weeks of treatment. Sarin, and sarin plus PB produced an average decrease in RBC AChE to 35% and 27% of pre-treatment respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

Delayed treatment effects.

Motor performance in the open field test: ANOVA was significant at 2 weeks after treatment for total distance moved within the arena. Multiple contrasts indicated that the sarin group mean was significantly lower than controls (Fig. 1). No difference vs. controls was found for the other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment.

ANOVA was also significant ($P<0.05$) at 2 weeks after treatment for the average distance to the arena's border. Multiple contrasts indicated that the sarin group mean (7.78 ± 0.39 cm) was significantly lower than PB (9.58 ± 0.45 cm), and sarin+PB (9.05 ± 0.45 cm), but not different from controls (8.63 ± 0.64 cm).

Nociceptive threshold: No statistically significant difference among groups was found for the flinch response to the test at 2 and 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher LSD test, $P<0.05$) showed that the nociceptive threshold of the animals that received the combination of sarin and PB was significantly higher than all other groups. ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response was significantly lower in the sarin group than in the PB, and sarin+PB groups, but not significantly different from controls. At 16 weeks after treatment, ANOVA was also significant and multiple comparisons showed that the sarin+PB group had a significantly higher threshold than all other groups. Data are presented in Fig. 2 for the jump response.

Reactivity (acoustic startle): A significant increase of sarin-treated animals against the controls in the average motor response over the 20 trials was observed in measurements performed 2 weeks after treatment. This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (Fig. 3). In this case, the mean of the sarin group was significantly higher than all others. No difference among group means was present at 4 or 16 weeks after treatment.

Cardiovascular regulation: Typical responses of BP and HR to phenylephrine and nitroprusside are shown in Fig 4. The highest phenylephrine dose elicited atrioventricular blockade (Fig 4, top) followed by nodal, and in some cases ventricular ectopic rhythms. The coefficient of the regression of HR on BP calculated from hypertension data prior to the A-V block yielded values similar to that of the regression obtained from hypotensive episodes. For that reason both sets of data were pooled in one analysis (Fig 5). In another analysis, only data from hypertensive episodes (including the A-V block) was used. None of the differences between experimental groups reached statistical significance.

Brain regional AChE and ChAT activities and QNB binding: Enzymatic activities were not affected at any time after any treatment, but QNB binding was reduced in hippocampus, caudate-putamen and mesencephalon, 2 weeks after exposure to sarin (data not shown). However, no changes were detected 4 and 16 weeks after treatments.

Discussion

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test two weeks after discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Russell, Booth, Lauretz, Smith, and Jenden, 1986;Overstreet, 1977). However, in the present experiments both blood and tissue ChE had recovered to normal levels at the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease particularly pronounced in caudate-putamen, hippocampus and mesencephalon. Downregulation of muscarinic receptors may have played a role in the behavioral phenomena described above since this was their only neurochemical correlate.

Both the depressed locomotor activity and enhanced startle response induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from sarin

lethality (Harris and Stitcher, 1984). Contrary to previous reports (Servatius et al., 1998), PB did not elicit delayed changes in acoustic startle.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperalgesia) in hypocholinergic states (Russell et al., 1990;Russell, Booth, Lauretz, Smith, and Jenden, 1986), and the reverse is true of hypercholinergic states (Shih and Romano, 1988). The facts the both the flinch and the jump response were enhanced only 16 weeks after treatment is difficult to interpret since neither cholinesterase activity nor cholinergic receptor binding were found altered at this time. Secondary delayed effects of the initial exposure to this drug combination may be at work and deserve further exploration.

The lack of changes in baseline levels of arterial blood pressure and heart rate as well as in the gain of the baroreceptor response are indications that the central and peripheral cholinergic steps involved in cardiovascular regulation were intact in the experimental groups under study.

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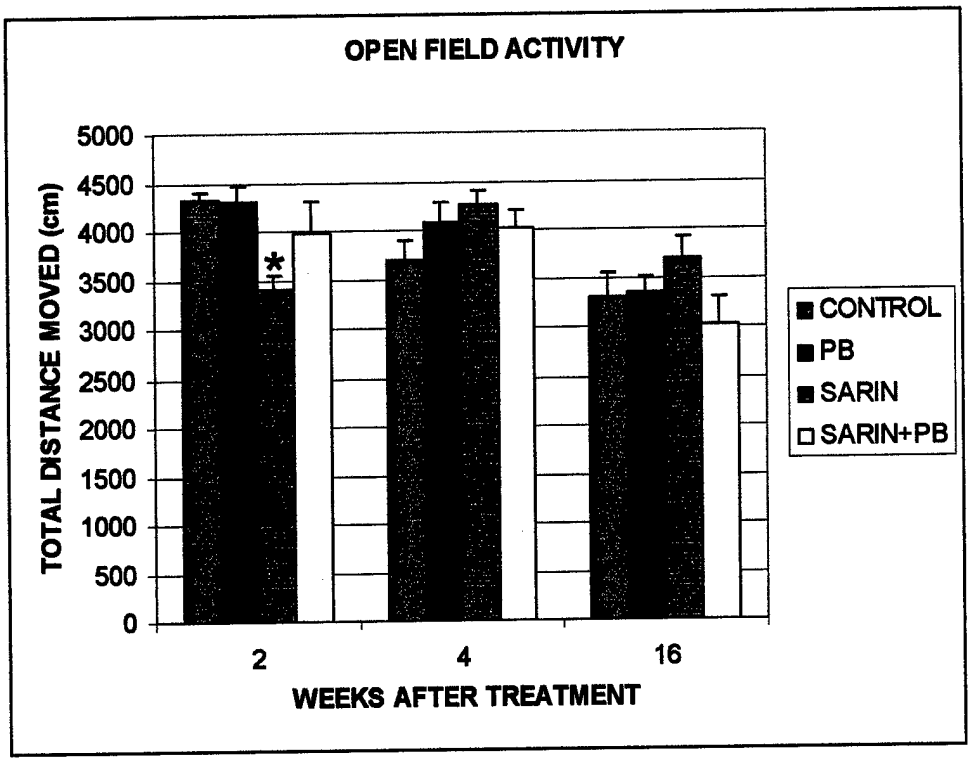

Figure 1: Means and SE of total distance moved in the open field for all experimental groups (12 rats per group). The sarin mean was significantly lower than controls at 2 weeks ($P < 0.05$, ANOVA and Fisher's multiple comparisons LSD test).

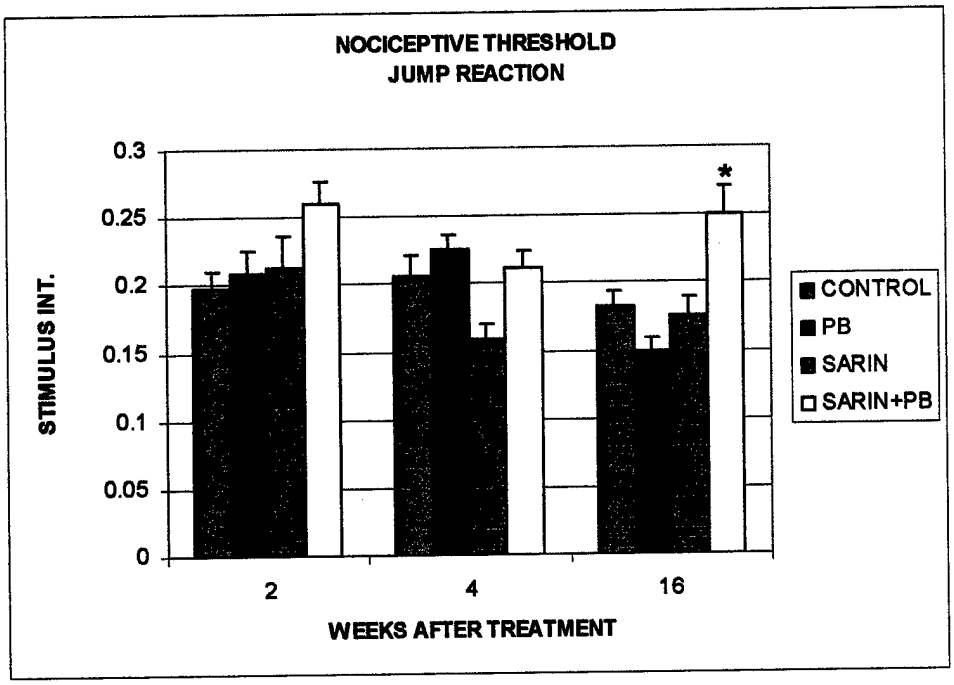

Figure 2: Means and SE of jump nociceptive threshold for all experimental groups (12 rats per group). The sarin+PB mean was significantly higher ($P < 0.05$, ANOVA and Fisher's multiple comparisons LSD test) than all others at 16 weeks post-treatment.

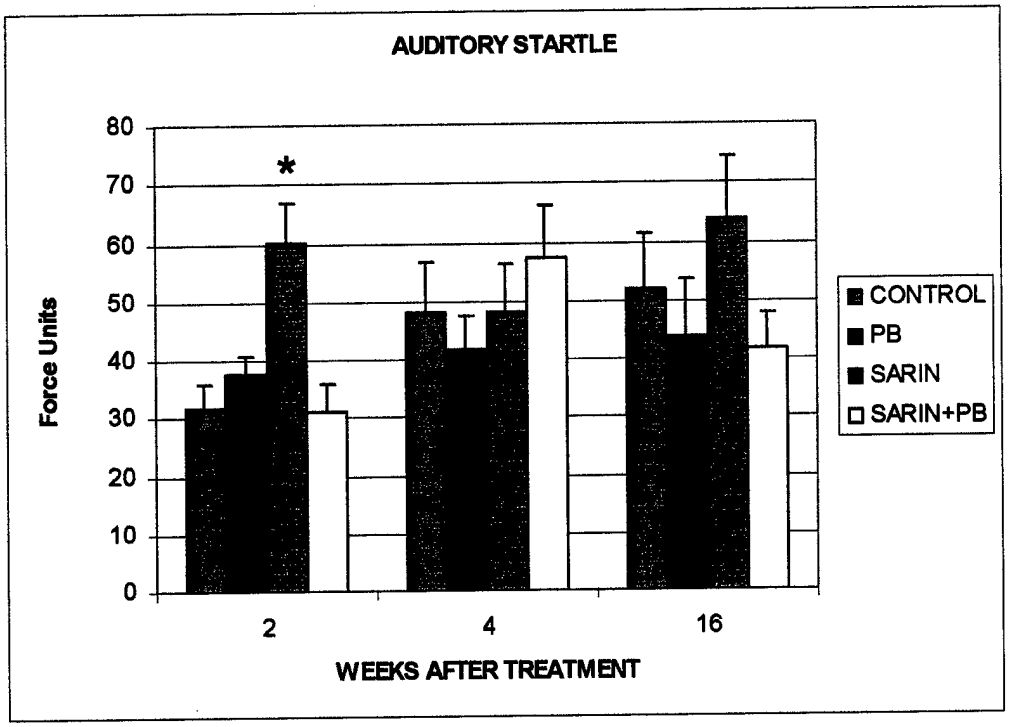

Figure 3: Means and SE of maximal response to acoustic startle for all experimental groups (12 rats per group). The sarin mean was higher than controls at 2 weeks after treatment ($P < 0.005$, ANOVA and Fisher's multiple comparisons LSD test).

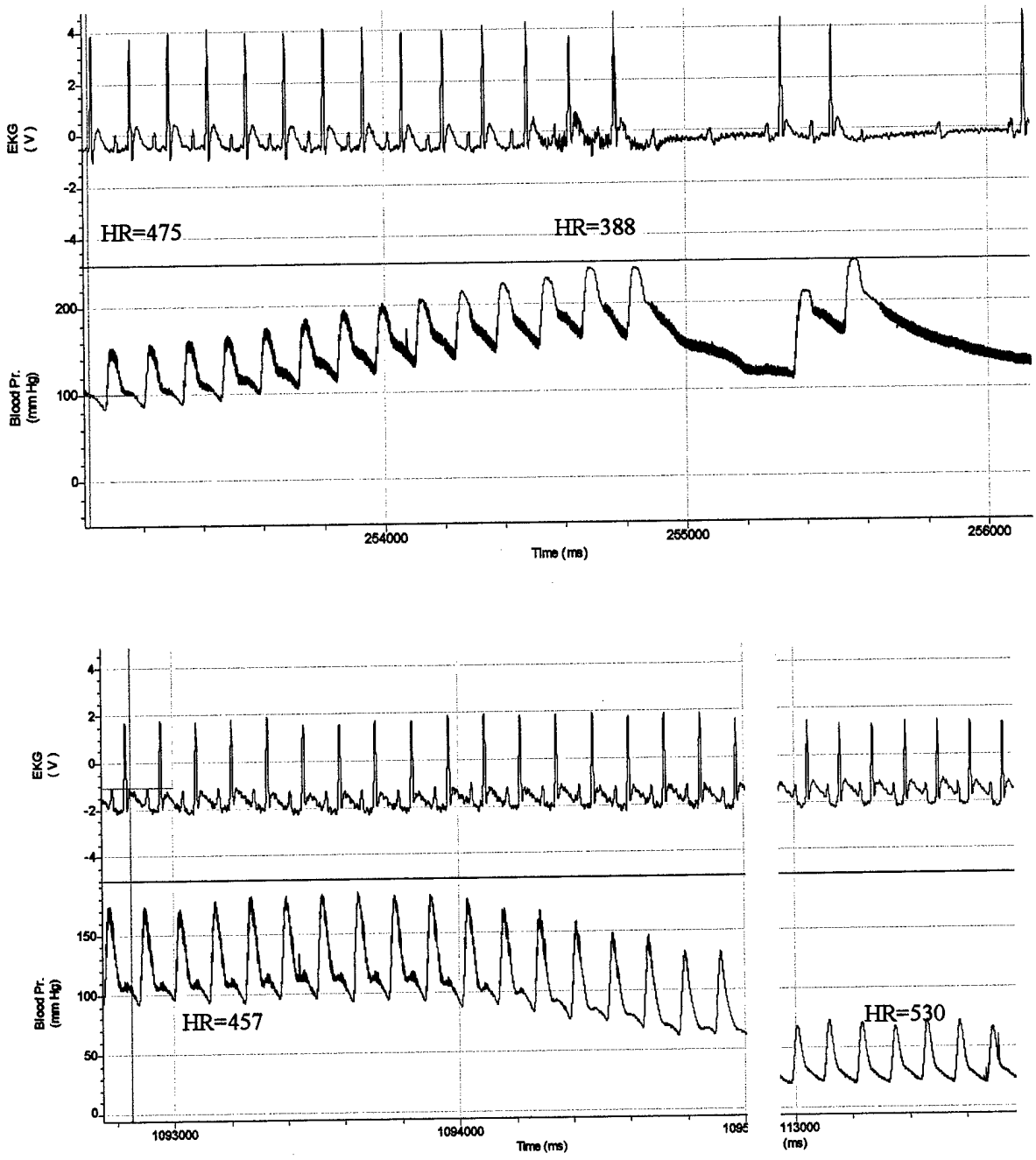

Figure 4:Representative baroreceptor mediated heart rate responses to pharmacologically induced hyper- or hypotension. TOP:Progressive hypertension and sinus bradycardia after phenylephrine (PE), followed by A-V block and nodal bygeminal rhythm. BOTTOM: Progressive hypotension and tachycardia following nitroprusside (NP).Two doses of each drug were given to every animal and the regression of HR on MABP calculated with or without inclusion of beats beyond the A-V block.

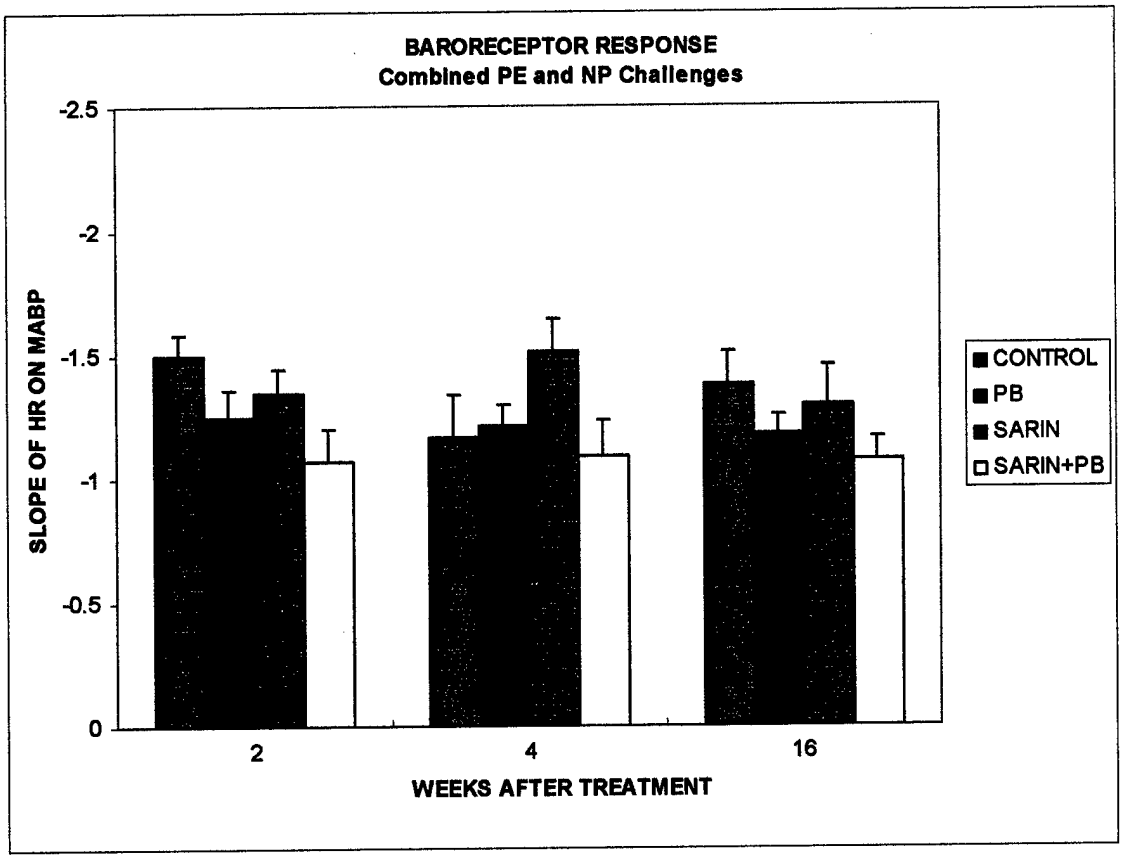

Figure 5: Mean and SE of slopes of the linear regression of HR on MABP for all PE and NP challenges excluding heart beats beyond the first episode of A-V block. None of the differences among means was statistically significant.